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L5	(1998)[PRAY] AND (l3)	0	L5
L4	@PRAY <= 1997 AND (l3)	2	L4
L3	L2 and (tumor or cancer)	80	L3
L2	L1 and (antisens$ or ribozym$)	96	L2
L1	sparc or osteonectin	2425	L1

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Search Results - Record(s) 1 through 2 of 2 returned.

1. Document ID: US 6331289 B1

L4: Entry 1 of 2 File: USPT Dec 18, 2001

US-PAT-NO: 6331289
DOCUMENT IDENTIFIER: US 6331289 B1

TITLE: Targeted diagnostic/therapeutic agents having more than one different vectors

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | Claims | KWIC | Draw Desc | Image

2. Document ID: US 5939270 A

L4: Entry 2 of 2 File: USPT Aug 17, 1999

US-PAT-NO: 5939270
DOCUMENT IDENTIFIER: US 5939270 A

TITLE: Markers for organ rejection

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | Claims | KWIC | Draw Desc | Image

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Term	Documents
@PRAY.USPT.	1113466
(3 AND ((@PRAY <= "1997")).USPT.	2
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L4: Entry 1 of 2 File: USPT Dec 18, 2001

DOCUMENT IDENTIFIER: US 6331289 B1
TITLE: Targeted diagnostic/therapeutic agents having more than one different vectors

Priority Application Year (1):
1996

Priority Application Year (2):
1996

Priority Application Year (3):
1997

Priority Application Year (4):
1997

Priority Application Year (5):
1997

Priority Application Year (6):
1997

Brief Summary Text (11):
There is also a number of publications concerning ultrasound contrast agents which refer in passing to possible use of monoclonal antibodies as vectors without giving significant practical detail and/or to reporters comprising materials which may be taken up by the reticuloendothelial system and thereby permit image enhancement of organs such as the liver--see, for example WO-A-9300933, WO-A-9401140, WO-A-9408627, WO-A-9428874, U.S. Pat. No. 5,088,499, U.S. Pat. No. 5,348,016 and U.S. Pat. No. 5,469,854. In general these prior art targeted contrast agents are intended to enhance contrast at specific sites in the body, for example tumour cells, by using one vector to bind strongly to one target, in order to achieve concentration at the target cells. In contrast to this principle of using one vector to bind with high affinity to one target, the present invention is based in part on the finding that diagnostic and/or therapeutically active agents with more favourable properties may be obtained by use of multiple kinds of vector-target interactions (e.g. involving agents associated with a plurality of different vectors and/or with one or more vectors having affinity for different targets on the same or different cell types). In this way, binding of gas-containing and gas-generating diagnostic and/or therapeutic agents may, for example, be obtained by forming multiple binding pairs between one vector with specificity for more than one receptor or between more than one vector with affinity for one or more types of target, with either low or high affinities. Such multiple binding of the vector-conjugated agent to one or more target molecules/structures may result in advantageous targeting properties, for example by enhancing target specificity and/or by distinguishing interactions at a desired target area from background interactions with lower levels of molecules/structures similar to target expressed elsewhere in the body.

Brief Summary Text (16):
The term multiple-specificity is also used to describe an injectable carrier liquid, of gas-containing or gas-generating material composed of one or more vectors with a specificity for one or more cellular surface receptors while at the same time comprising a second element with specificity for a substrate or receptor system

binding to which induces a therapeutic response. Thus included within the scope of the present invention are multiple specific imaging agents comprising a targeting vector, such as the anti fibrin antibody described by Lanza et al. Circulation, 1996 94 (12),pp 3334 , annexin V atherosclerotic plaque binding peptides such as YRALVDTLK, or any other vector known to associate with fibrin clots, in combination with a drug or enzyme with fibrinolytic activity such as streptokinase, plasminogen activator (tPA), urokinase (uPA) or prourokinase (scuPA) resulting in a localised therapeutic antithrombotic effect. This invention is also extended to include vectors with increased specificity for tumour cells in combination with vectors or drug molecules functioning as chemotherapeutic agents capable of inhibiting tumour growth.

Brief Summary Text (17):
It is well known that many, if not all, target molecules are not expressed exclusively at target sites; a common situation is that such molecules are over-expressed by target cells or at a target structure but are also expressed at lower levels elsewhere in the body. The use of reporters carrying a multiplicity of vectors with relatively low affinity for the target may be advantageous in this situation, since the reporter will then tend to concentrate in regions of high target density which permit multiple (and therefore strong) binding to the reporter (e.g. a gas-containing agent incorporating the vectors folic acid and glutathione for multiple-specific binding to folic acid receptors and glutathione S-trasferase receptors respectively which are over-expressed as tumour cells). Areas of low target density, on the other hand, will not provide sufficient interaction with such low affinity vectors to bind the target. In such embodiments of the invention, low affinity vectors may be regarded as having an association constant K.sub.a for interaction with a target molecule or structure of less than 10.sup.8 M.sup.-1, e.g. less than 10.sup.7 M.sup.-1, preferably less than 10.sup.6 M.sup.-1. A further embodiment of this invention is thus based on the finding that the desired binding of gas-containing and gas-generating diagnostic and/or therapeutic agents may be obtained by forming binding pairs with low affinity between more than one type of vector and one or more type of target. Multiple vectors may therfore be used to increase specificity, so that the reporter will bind only to target cells or structures expressing a particular combination of target molecules.

Brief Summary Text (19):
Products comprising two or more vectors with different specificities, i.e. which bind to different target molecules on different cells, may advantageously be used as "general purpose" agents for detection of a range of diseases, e.g. different forms of cancer. Thus, for example, the use of such agents may enable detection of metastases, which are often heterogeneous with respect to expression of target molecules (i.e. antigens).

Detailed Description Text (25):
viii) aziridines based on s-triazine compounds detailed above, e.g. as described by Ross, W. C. J. in Adv. Cancer Res. (1954) 2, 1, which react with nucleophiles such as amino groups by ring opening;

Detailed Description Text (75):
xii) polyanhydrides such as copolymers of sebacic acid (octanedioic acid) with bis(4-carboxy-phenoxy)propane, which have been shown in rabbit studies (see Brem, H., Kader, A., Epstein, J. I., Tamargo, R. J., Domb, A., Langer, R. and Leong, K. W. in Sel. Cancer Ther. (1989) 5, 55-65) and rat studies (see Tamargo, R. J., Epstein, J. I., Reinhard, C. S., Chasin, M. and Brem, H. in J. Biomed. Mater. Res. (1989) 23, 253-266) to be useful for controlled release of drugs in the brain without evident toxic effects;

Detailed Description Text (88):
antineoplastic agents such as vincristine, vinblastine, vindesine, busulfan, chlorambucil, spiroplatin, cisplatin, carboplatin, methotrexate, adriamycin, mitomycin, bleomycin, cytosine arabinoside, arabinosyl adenine, mercaptopurine, mitotane, procarbazine, dactinomycin (antinomycin D), daunorubicin, doxorubicin hydrochloride, taxol, plicamycin, aminoglutethimide, estramustine, flutamide, leuprolide, megestrol acetate, tamoxifen, testolactone, trilostane, amsacrine (m-AMSA), asparaginase (L asparaginase), etoposide, interferon a-2a and 2b, blood products such as hematoporphyrins or derivatives of the foregoing; biological response modifiers such as muramylpeptides; antifungal agents such as ketoconazole, nystatin,

griseofulvin, flucytosine, miconazole or amphotericin B; hormones or hormone analogues such as growth hormone, melanocyte stimulating hormone, estradiol, beclomethasone dipropionate, betamethasone, cortisone acetate, dexamethasone, flunisolide, hydrocortisone, methylprednisolone, paramethasone acetate, prednisolone, prednisone, triamcinolone or fluorocortisone acetate; vitamins such as cyanocobalamin or retinoids; enzymes such as alkaline phosphatase or manganese superoxide dismutase; antiallergic agents such as amelexanox; inhibitors of tissue factor such as monoclonal antibodies and Fab fragments thereof, synthetic peptides, nonpeptides and compounds downregulating tissue factor expression; inhibitors of platelets such as, GPIa, GPIb and GPIIb-IIIa, ADP receptors, thrombin receptors, von Willebrand factor, prostaglandins, aspirin, ticlopidin, clopigogrel and reopro; inhibitors of coagulation protein targets such as: FIIa FVa, FVIIa, FVIIIA, FIXa, tissue factor, heparins, hirudin, hirulog, argatroban, DEGR rFVIIa and annexin V; inhibitors of fibrin formation and promoters of fibrinolysis such as t-PA, urokinase, Plamin, Streptokinase, rt-Plasminogen Activator and rStaphylokinase; antiangiogenic factors such as medroxyprogesteron, pentosan polysulphate, suramin, taxol, thalidomide, angiostatin, interferon-alpha, metalloproteinase inhibitors, platelet factor 4, somatostatin, thromobospondin; circulatory drugs such as propranolol; metabolic potentiators such as glutathione; antituberculars such as p-aminosalicylic acid, isoniazid, capreomycin sulfate, cyclosexine, ethambutol, ethionamide, pyrazinamide, rifampin or streptomycin sulphate; antivirals such as acyclovir, amantadine, azidothymidine, ribavirin or vidarabine; blood vessel dilating agents such as diltiazem, nifedipine, verapamil, erythritol tetranitrate, isosorbide dinitrate, nitroglycerin or pentaerythritol tetranitrate; antibiotics such as dapsone, chloramphenicol, neomycin, cefaclor, cefadroxil, cephalexin, cephradine, erythromycin, clindamycin, lincomycin, amoxicillin, ampicillin, bacampicillin, carbenicillin, dicloxacillin, cyclacillin, picloxacillin, hetacillin, methicillin, nafcillin, penicillin, polymyxin or tetracycline; antiinflammatories such as diflunisal, ibuprofen, indomethacin, meclefenamate, mefenamic acid, naproxen, phenylbutazone, piroxicam, tolmetin, aspirin or salicylates; antiprotozoans such as chloroquine, metronidazole, quinine or meglumine antimonate; antirheumatics such as penicillamine; narcotics such as paregoric; opiates such as codeine, morphine or opium; cardiac glycosides such as deslaneside, digitoxin, digoxin, digitalin or digitalis; neuromuscular blockers such as atracurium mesylate, gallamine triethiodide, hexafluorenium bromide, metocurine iodide, pancuronium bromide, succinylcholine chloride, tubocurarine chloride or vecuronium bromide; sedatives such as amobarbital, amobarbital sodium, aprobbarbital, butabarbital sodium, chloral hydrate, ethchlorvynol, ethinamate, flurazepam hydrochloride, glutethimide, methotrimeprazine hydrochloride, methyprylon, midazolam hydrochloride, paraldehyde, pentobarbital, secobarbital sodium, talbutal, temazepam or triazolam; local anaesthetics such as bupivacaine, chloroprocaine, etidocaine, lidocaine, mepivacaine, procaine or tetracaine; general anaesthetics such as droperidol, etomidate, fentanyl citrate with droperidol, ketamine hydrochloride, methohexital sodium or thiopental and pharmaceutically acceptable salts (e.g. acid addition salts such as the hydrochloride or hydrobromide or base salts such as sodium, calcium or magnesium salts) or derivatives (e.g. acetates) thereof. Other examples of therapeutics include genetic material such as nucleic acids, RNA, and DNA of natural or synthetic origin, including recombinant RNA and DNA. DNA encoding certain proteins may be used in the treatment of many different types of diseases. For example, tumor necrosis factor or interleukin-2 genes may be provided to treat advanced cancers; thymidine kinase genes may be provided to treat ovarian cancer or brain tumors; interleukin-2 genes may be provided to treat neuroblastoma, malignant melanoma or kidney cancer; and interleukin-4 genes may be provided to treat cancer.

Detailed Description Text (91):
Tumors must initiate angiogenesis when they reach millimeter size in order to keep up their rate of growth. As angiogenesis is accompanied by characteristic changes in the endothelial cells and their environment, this process is a promising target for therapeutic intervention. The transformations accompanying angiogenesis are also very promising for diagnosis, a preferred example being malignant disease, but the concept also shows great promise in inflammation and a variety of inflammation-related diseases. These factors are also involved in re-vascularisation of infarcted parts of the myocardium, which occurs if a stenosis is released within a short time.

Detailed Description Text (95):

Another alternative is to incorporate the prodrug, the prodrug activating enzyme and the vector in the same microbubble in a system where the prodrug will only be activated after some external stimulus. Such a stimulus may, for example, be a tumour specific protease as described above, or bursting of the bubbles by external ultrasound after the desired targeting has been achieved.

Detailed Description Text (99):
i) Antibodies, which can be used as vectors for a very wide range of targets, and which have advantageous properties such as very high specificity, high affinity (if desired), the possiblity of modifying affinity according to need etc. Whether or not antibodies will be bioactive will depend on the specific vector/target combination. Both conventional and genetically engineered antibodies may be employed, the latter permitting engineering of antibodies to particular needs, e.g. as regards affinity and specificity. The use of human antibodies may be preferred to avoid possible immune reactions against the vector molecule. A further useful class of antibodies comprises so-called bispecific antibodies, i.e. antibodies having specificity for two different target molecules in one antibody molecule. Such antibodies may, for example, be useful in promoting formation of bubble clusters and may also be used for various therapeutic purposes, e.g. for carrying toxic moieties to the target. Various aspects of bispecific antibodies are described by McGuinness, B. T. et al. in Nat. Biotechnol. (1996) 14, 1149-1154; by George, A. J. et al. in J. Immunol. (1994) 152, 1802-1811; by Bonardi et al. in Cancer Res. (1993) 53, 3015-3021; and by French, R. R. et al. in Cancer Res. (1991) 51, 2353-2361.

Detailed Description Text (102):
iv) Oligonucleotides and modified oligonucleotides which bind DNA or RNA through Watson-Crick or other types of base-pairing. DNA is usually only present in extracellular space as a consequence of cell damage, so that such oligonucleotides, which will usually be non-bioactive, may be useful in, for example, targeting of necrotic regions, which are associated with many different pathological conditions. Oligonucleotides may also be designed to bind to specific DNA- or RNA-binding proteins, for example transcription factors which are very often highly overexpressed or activated in tumour cells or in activated immune or endothelial cells. Combinatorial libraries may be used to select oligonucleotides which bind specifically to possible target molecules (from proteins to caffeine) and which therefore may be employed as vectors for targeting.

Detailed Description Text (108):
Other peptide vectors and lipopeptides thereof of particular interest for targeted ultrasound imaging are listed below: Atherosclerotic plaque binding peptides such as YRALVDTLK (SEQ ID NO:22), YAKFRETLEDTRDRMY (SEQ ID NO:23) and RALVDTEFKVKQEAGAK (SEQ ID NO:24); Thrombus binding peptides such as NDGDFEEIPEEYLQ (SEQ ID NO:25) and GPRG (SEQ ID NO:26); Platelet binding peptides such as PLYKKIIKKLLES (SEQ ID NO:27); and cholecystokinin, .alpha.-melanocyte-stimulating hormone, heat stable enterotoxin 1, vasoactive intestinal peptide, synthetic alpha-M2 peptide from the third heavy chain complementarity-determining region and analogues thereof for tumor targeting.

Detailed Description Text (110):
a.) Heider, K. H., M. Sproll, S. Susani, E. Patzelt, P. Beaumier, E. Ostermann, H. Ahorn, and G. R. Adolf. 1996. "Characterization of a high-affinity monoclonal antibody specific for CD44v6 as candidate for immunotherapy of squamous cell carcinomas". Cancer Immunology Immunotherapy 43: 245-253.

Detailed Description Text (115):
B. Barinaga, M. 1997. "Designing Therapies That Target Tumor Blood Vessels". Science 275 (Jan. 24): 482-484.

Detailed Description Text (117):
D. Fox, S. B., and A. L. Harris. 1997. "Markers of tumor angiogenesis: Clinical applications in prognosis and anti-angiogenic therapy". Investigational New Drugs 15 (1): 15-28.

Detailed Description Text (118):
E. Gastl, G., T. Hermann, M. Steurer, J. Zmija, E. Gunsilius, J. Unger, and A. Kraft. May 1997. "Angiogenesis as a target for tumor treatment". Oncology 54 (3): 177-184.

Detailed Description Text (119):
F. Griffioen, A. W., M. J. H. Coenen, C. A. Damen, S. M. M. Hellwig, D. H. J. Vanweering, W. Vooys, G. H. Blijham, and G. Groenewegen. 1 August 1997. "CD44 is involved in tumor angiogenesis; an activation antigen on human endothelial cells". Blood 90 (3): 1150-1159.

Detailed Description Text (120):
G. Hlatky, L., P. Hahnfeldt, and C. N. Coleman. 1996. "Vacular endothelial growth factor: environmental controls and effects in angiogenesis". Brit. J. Cancer 74 Suppl. XXVII: S151-S156.

Detailed Description Text (122):
I. Nguyen, M. 1997. "Angiogenic factors as tumor markers". Investigational New Drugs 15 (1): 29-37.

Detailed Description Text (123):
J. Ono, M., H. Izumi, S. Yoshida, D. Gtot, S. Jimi, N. Kawahara, T. Shono, S. Ushiro, M. Ryoto, K. Kohno, Y. Sato, and M. Kuwano. 1996. "Angiogenesis as a new target for cancer treatment". Cancer Chemoter. Pharmacol. 38 (Suppl.): S78-S82.

Detailed Description Text (124):
K. Passe, T. J., D. A. Bluemke, and S. S. Siegelman. June 1997. "Tumor angiogenesis: Tutorial on implications for imaging". Radiology 203 (3): 593-600.

Detailed Description Text (125):
L. Saclarides, T. J. February 1997. "Angiogenesis in colorectal cancer". Surgical Clinics of North America 77 (1): 253.

Detailed Description Text (127):
N. Sagi-Assif, O., A. Traister, B. Z. Katz, R. Anavi, M. Eskenazy, and I. P. Witz. 1996. "TNF.alpha. and anti-Fas antibodies regulate Ly-6E.1 expression by tumor cells: A possible link between angiogenesis and Ly-6E.1". Immunology Letters 54: 207-213.

Detailed Description Text (128):
O. Strawn, L. M., G. McMahon, H. App, R. Schreck, W. R. Kuchler, M. P. Longhi, T. H. Hui, C. Tang, A. Levitzki, A. Gazit, I. Chen, G. Keri, L. Orfi, W. Risau, I. Flamme, A. Ullirch, K. P. Hirth, and L. K. Shawyer. 1996. "Flk-1 as a Target for Tumor Growth Inhibition". Cancer Res. 56: 3540-3545.

Detailed Description Text (132):
S. Yoshida, O. M., T. Shono, H. Izumi, T. Ishibashi, H. Suzuki, and M. Kuwano. 1997. "Involvement of Interleukin-8, Vascular Endothelial Growth Factor, and Basic Fibroblast Growth Factor in Tumor Necrosis Factor Alpha-Dependent Angiogenesis". Mol. Cell. Biol. 17: 4015-4023.

Detailed Description Text (133):
T. Zimrin, A. B., M. S. Pepper, G. A. McMahon, F. Nguyen, R. Montesano, and T. Maciag. 1996. "An Antisense Oligonucleotide to the Notch Ligand Jagged Enhances Fibroblast Growth Factor-induced Angiogenesis<in vitro>". J. Biol. Chem. 271 (Dec. 20): 32499-3502.

Detailed Description Text (138):
Z. Folkman, J. 1996. Tumor angiogenesis and tissue factor. Nature Medicine 2, 167-8

Detailed Description Text (139):
ZA. Relf, M., S. LeJeune, P.A. Scott, S. Fox, K. Smith, R. Leek, A. Moghaddam, R. Whitehouse, R. Bicknell and A.L. Harris. 1997. "Expression of the angiogenic factors vascular endothelial cell growth factor, acidic and basic fibroblast growth factor, tumor growth factor beta-1, platelet-derived endothelial cell growth factor, placenta growth factor and pleiotrophin in human primary breast cancer and its relation to angiogenesis". Cancer Res. 57, 963-9.

Detailed Description Text (343):
It is also considered relevant that the disulphide bond linking the methotrexate

structure to the microbubble may be reduced in vivo liberating the free drug molecule. This in combination with a tumour specific vector is a drug delivery system. A physiologically relevant reducing agent such as glutathione may be used to bring about drug release.

Detailed Description Text (405):
In combination with a tumour specific vector these microbubbles are considered useful as targeted drug delivery agents.

Detailed Description Paragraph Table (5):
Protein and peptide vectors - antibodies Vector type Receptor Comments/areas of use Ref antibodies (general) CD34 vascular diseases in general, normal vessel wall (e.g myocardium), activated endothelium, immune cells " ICAM-1 vascular diseases in general, normal vessel wall (e.g myocardium), activated endothelium, immune cells " ICAM-2 vascular diseases in general, normal vessel wall (e.g myocardium), activated endothelium, immune cells " ICAM-3 vascular diseases in general, normal vessel wall (e.g myocardium), activated endothelium, immune cells " E-selectin vascular diseases in general, normal vessel wall (e.g myocardium), activated endothelium, immune cells " P-selectin vascular diseases in general, normal vessel wall (e.g myocardium), activated endothelium, immune cells " PECAM vascular diseases in general, normal vessel wall (e.g myocardium), activated endothelium, immune cells " Integrins, vascular diseases in general, e.g. VLA-1, normal vessel wall (e.g VLA-2, VLA-3 myocardium), activated VLA-4, VLA-5, endothelium, immune cells VLA-6, .beta..sub.1.alpha..sub.7, .beta..sub.1.alpha..sub.8, .beta..sub.1.alpha..sub.v, LFA-1, Mac-1, CD41a, etc. " GlyCAM Vessel wall in lymph nodes (quite specific for lymph nodes) " MadCam 1 Vessel wall in lymph nodes (quite specific for lymph nodes) " fibrin Thrombi " Tissue Activated endothelium, Factor tumours " Myosin Necrosis, myocardial infaction " CEA Tumours (carcinoem bryonal antigen) " Mucins Tumours " Multiple drug Tumours resistance protein Prostate Prostate cancer specific antigen " Cathepsin B Tumours (proteases of various kinds are often more or less specifically overexpressed in a variety of tumours - Cathepsin B is such a protease) " Transferrin Tumors, receptor vessel wall MoAb 9.2.27 Tumours Antigen upregulated on cell growth VAP-1 Adhesion molecule Band 3 Upregulated during phagocytic protein activity CD44 tumor cells .beta.2- general microglobulin MHC class I general antibody integrin tumors, angiogenisis c .alpha.v.beta.3 antibodies CD44 tumour cells a antibodies .beta.2- general b microglobulin antibodies MHC class I general b

Detailed Description Paragraph Table (7):
Vectors comprising cytokines/growth factors/peptide hormones and fragments thereof Vector type Receptor Comments/areas of use Ref Epidermal growth EGF-receptor or Tumours factor related receptors Nerve growth NGF-receptor Tumours factor Somatostatin ST-receptor Tumours Endothelin Endothelin- Vessel wall receptor Interleukin-1 IL-1-receptor Inflammation, activated cells of different kinds Interleukin-2 IL-2-receptor Inflammation, activated cells of different kinds Chemokines (ca. Chemokine Inflammation 20 different receptors, cytokines partly proteoglycans sharing receptors) Tumour necrosis TNF-receptors Inflammation factor Parathyroid PTH-receptors Bone diseases hormone Kidney diseases Bone BMP-receptors Bone Diseases Morphogenetic Protein Calcitonin CT-receptors Bone diseases Colony Corresponding Endothelium stimulating specific factors (G-CSF, receptors, GM-CSF, M-CSF, proteoglycans IL-3) Insulin like IGF-I receptor Tumours, growth factor I other growing tissues Atrial ANF-receptors Kidney, Natriuretic vessel wall Factor Vasopressin Vasopressin Kidney, receptor vessel wall VEGF VEGF-receptor Endothelium, regions of angiogenesis Fibroblast FGF-receptors, Endothelium growth factors Proteoglycans Angiogenesis Schwann cell proteoglycans growth factor specific receptors

Detailed Description Paragraph Table (8):
Miscellaneous protein and peptide vectors Vector type Receptor Comments/areas of use Ref Streptavidin Kidney Kidney diseases Bacterial Fibronectin Vessel wall fibronectin-binding proteins Fc-part of Fc-receptors Monocytes antibodies macrophages liver Transferrin transferrin- Tumours receptor vessel walls Streptokinase/ thrombi thrombi tissue plasminogen activator Plasminogen, Fibrin Thrombi, plasmin tumours Mast cell proteoglycans proteinases Elastase proteoglycans Lipoprotein proteoglycans lipase Coagulation proteoglycans enzymes Extracellular proteoglycans superoxide dismutase Heparin cofactor proteoglycans II Retinal survival proteoglycans factor specific receptors Heparin binding proteoglycans brain mitogen specific receptors

Apolipoprotein, proteoglycans e.g. specific apolipoprotein B receptors e.g., LDL receptor Apolipoprotein E LDL receptor proteoglycans Adhesion- proteoglycans promoting proteins, e.g. Purpurin Viral coat proteoglycans proteins, e.g. HIV, Herpes Microbial "Antigen 85" fibronectin, collagen, adhesins complex of fibrinogen, vitronectin, mycobacteria heparan sulfate .beta. amyloid proteoglycans .beta.-amyloid accumulates in precursor Alzheimer's disease Tenascin, heparan sulfate, e.g. tenascin C integrins

Detailed Description Paragraph Table (10):
Vectors comprising anti angiogenic factors Vector type Target Comments areas of use Ref Angiostatin EC of tumors plasminogen fragment F cartilage-derived EC of tumors J inhibitor .beta. Cyclodextrin tumors, C tetradecasulfate inflammation fumagillin and analogs tumors, E inflammation Interferon-.alpha. EC of tumors K Interferon-.gamma. EC of tumors E interleukin 12 EC of tumors E linomide tumors, A inflammation medroxyprogesterone EC of tumors K metalloproteinase EC of tumors K inhibitors pentosan polysulfate EC of tumors K platelet factor 4 EC of tumors M Somatostatin EC of tumors K Suramin EC of tumors K Taxol EC of tumors K thalidomide EC of tumors K Thrombospondin EC of tumors K

Detailed Description Paragraph Table (11):
Vectors comprising angiogenic factors Comments/areas Vector type Target of use Ref acidic fibroblast growth EC of tumors K factor adenosine EC of tumors K Angiogenin EC of tumors K Angiotensin II EC of tumors K basement membrane tumors e.g., tenascin, M components collagen IV basic fibroblast growth EC of tumors K factor Bradykinin EC of tumors K Calcitonin gene-related EC of tumors K peptide epidermal growth factor EC of tumors K Fibrin tumors K Fibrinogen tumors K Heparin EC of tumors K histamine EC of tumors K hyaluronic acid or fragments EC of tumors K thereof Interleukin-1.alpha. EC of tumors K laminin, laminin fragments EC of tumors K nicotinamide EC of tumors K platelet activating factor EC of tumors K platelet-derived endothelial EC of tumors K growth factor prostaglandins E1, E2 EC of tumors K spermine EC of tumors K spermine EC of tumors K Substance P EC of tumors K transforming growth factor-.alpha. EC of tumors K transforming growth factor-.beta. EC of tumors K Tumor necrosis factor-.beta. EC of tumors K vascular endothelial growth EC of tumors K factor/vascular permeability factor vitronectin A

Detailed Description Paragraph Table (12):
Vector molecules other than recognized angiogenetic factors with known affinity for receptors associated with angiogenesis Vector type Target Comments/areas of use Ref angiopoietin tumors, E inflammation .alpha..sub.2 -antiplasmin tumors, inflammation combinatorial tumors, for instance: compounds libraries, compounds inflammation that bind to basement from membrane after degradation endoglin tumors, D inflammation endosialin tumors, D inflammation endostatin [collagen tumors, M fragment] inflammation Factor VII related tumors, D antigen inflammation fibrinopeptides tumors, ZC inflammation fibroblast growth tumors, E factor, basic inflammation hepatocyte growth tumors, I factor inflammation insulin like growth tumors, R factor inflammation interleukins tumors, e.g.,: IL-8 I inflammation leukemia inhibitory tumors, A factor inflammation metalloproteinase tumors, e.g., batimastat E inhibitors inflammation Monoclonal antibodies tumors, for instance: to inflammation angiogenetic factors or their receptors, or to components of the fibrinolytic system peptides, for instance tumors, B, Q cyclic RGD.sub.D FV inflammation placental growth factor tumors, J inflammation placental tumors, E proliferin-related inflammation protein plasminogen tumors, M inflammation plasminogen activators tumors, D inflammation plasminogen activator tumors, U, V inhibitors inflammation platelet activating tumors, inhibitors of angiogenesis A factor antagonists inflammation platelet-derived growth tumors, E factor inflammation pleiotropin tumors, ZA inflammation proliferin tumors, E inflammation proliferin related tumors, E protein inflammation selectins tumors, e.g., E-selectin D inflammation SPARC tumors M inflammation snake venoms tumors, Q (RGD-containing) inflammation Tissue inhibitor of tumors, e.g., TIMP-2 U metalloproteinases inflammation thrombin tumors, H inflammation thrombin-receptor-tumors, H activating tetra- inflammation decapeptide thymidine tumors, D phosphorylase inflammation tumor growth factor tumors, ZA inflammation

Detailed Description Paragraph Table (13):
Receptors targets associated with angiogenesis Vector type Target Comments/areas of use Ref biglycan tumors, dermatan sulfate X inflammation proteoglycan CD34 tumors, L

inflammation CD44 tumors, F inflammation collagen type I, IV, tumors, A VI, VIII inflammation decorin tumors, dermatan sulfate V inflammation proteoglycan dermatan sulfate tumors, X proteoglycans inflammation endothelin tumors, G inflammation endothelin receptors tumors, G inflammation fibronectin tumors P Flk-1 KDR, Flt-4 tumors, VEGF receptor D inflammation FLT-1 (fms-like tumors, VEGF-A receptor C tyrosine kinase) inflammation heparan sulfate tumors, P inflammation hepatocyte growth tumors, I factor receptor (c-met) inflammation insulin-like growth tumors, R factor mannose-6- inflammation phosphate receptor integrins: Tumors, D, .beta..sub.3 and .beta..sub.5, inflammation P integrin .alpha..sub.V.beta..sub.3, integrin .alpha..sub.6.beta..sub.1, laminin receptor integrins .alpha..sub.6, integrins .beta..sub.1, integrin .alpha..sub.2.beta..sub.1, integrin .alpha..sub.V.beta..sub.3, integrin .alpha..sub.5 subunit of the fibronectin receptor integrin .alpha..sub.V.beta..sub.5, fibrin receptors. Intercellular adhesion tumors, F molecule-1 and -2 inflammation Jagged gene product tumors, T inflammation Ly-6 tumors, a lymphocyte activation N inflammation protein matrix tumors, D metalloproteinases inflammation MHC class II tumors, inflammation Notch gene product tumors, T inflammation Osteopontin tumors Z PECAM tumors, alias CD31 P inflammation plasminogen activator tumors, CC receptor inflammation platelet-derived growth tumors, E factor receptors inflammation Selectins: E-, P- tumors, D inflammation Sialyl Lewis-X tumors, blood group antigen M inflammation stress proteins: tumors, molecular chaperones glucose regulated, inflammation heat shock families and others syndecan tumors, T inflammation thrombospondin tumors, M inflammation TIE receptors tumors, tyrosine kinases with Ig- E inflammation and EGF-like domains tissue factor tumors, Z inflammation tissue inhibitor of tumors, e.g., TIMP-2 U metalloproteinases inflammation transforming growth tumors, E factor receptor inflammation urokinase-type tumors, D plasminogen activator inflammation receptor Vascular cellular tumors, D adhesion molecule inflammation (VCAM) Vascular endothelial tumors, growth factor related inflammation protein Vascular endothelial tumors, F growth factor-A inflammation receptor von Willebrand factor- tumors, L related antigen inflammation

Detailed Description Paragraph Table (14):
Oligonucleotide vectors Vector type Receptor Comments/areas of use Ref Oligonucleotides DNA made Tumours complementary to available by Myocardial infection repeated necrosis All other diseases that sequences, e.g. involves necrosis genes for ribosomal RNA, Alu sequences Oligonucleotides DNA made Tumours complementary to available by disease-specific necrosis in a mutations (e.g. region of the mutated relevant disease oncogenes) Oligonucleotides DNA of infective Viral or bacterial complementary to agent infections DNA of infecting agent. Triple or As in above As in above examples quadruple-helix examples forming oligonucleotides Oligonucleotides DNA-binding Tumours with recognition protein, e.g. Activated endothelium sequence for transcription Activated immune cells DNA- or RNA- factors (often binding proteins overexpressed/ activated in tumours or activated endothelium/ immune cells

Detailed Description Paragraph Table (17):
Receptors comprising DNA-binding drugs Vector type Receptor Comments/areas of use Ref acridine DNA made Tumours, derivatives available by Myocardial infarction and distamycin necrosis all other diseases involving netropsin necrosis or other processes actinomycin D liberating DNA from cells echinomycin bleomycin etc.

Detailed Description Paragraph Table (18):
Receptors comprising DNA-binding drugs Vector type Receptor Comments/areas of use Ref acridine DNA made Tumours, derivatives available by Myocardial infarction and distamycin necrosis all other diseases involving netropsin necrosis or other processes actinomycin D liberating DNA from cells echinomycin bleomycin etc.

Detailed Description Paragraph Table (19):
Vectors from combinatorial libraries Vector type Receptor Comments/areas of use Ref Antibodies with Any of above Any diseased or normal structure targets - or may structure of interest, e.g. determined be unknown when thrombi, tumors or walls of during make functional myocardial vessels generation selection of process vector binding to chosen diseased structure Peptides with Any of above Any diseased or normal sequence targets - or may structure of interest, e.g. determined be unknown when thrombi, tumours or walls of during make functional myocardial vessels generation selection of process vector binding to chosen diseased structure Oligonucleotides Any of above Any diseased or normal with sequence targets - or may structure of interest,

e.g. determined be unknown when thrombi, tumours or walls of during make functional myocardial vessels generation selection of process vector binding to chosen diseased structure Modifications of Any of above Any diseased or normal oligos obtained targets , or may structure of interest, e.g. as above be unknown when thrombi, tumours or walls of make functional myocardial vessels selection of vector binding to chosen diseased structure Other chemicals Any of above Any diseased or normal with structure targets , or may structure of interest, e.g. determined be unknown when thrombi, tumours or walls of during make functional myocardial vessels generation selection of process vector binding to chosen diseased structure

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$0.01 Estimated total session cost 0.223 DialUnits

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File 155:MEDLINE(R) 1966-2003/Feb W4
(c) format only 2003 The Dialog Corp.
File 5:Biosis Previews(R) 1969-2003/Feb W4
(c) 2003 BIOSIS
*File 5: Alert feature enhanced for multiple files, duplicates removal, customized scheduling. See HELP ALERT.
File 444:New England Journal of Med. 1985-2003/Mar W1
(c) 2003 Mass. Med. Soc.

Set	Items	Description

? s bone and tissue and review and py>1999

	651244	BONE
	1269505	TISSUE
	621121	REVIEW
	3220621	PY>1999
S1	1025	BONE AND TISSUE AND REVIEW AND PY>1999

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	160501	MINERAL?
S2	76	S1 AND MINERAL?

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...examined 50 records (50)
...completed examining records

S3	66	RD (unique items)

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3/3,AB/1 (Item 1 from file: 155)
DIALOG(R)File 155:MEDLINE(R)

14467171 22479160 PMID: 12590987
Bisphosphonate mechanism of action.
Reszka Alfred A; Rodan Gideon A
Department of Bone Biology and Osteoporosis Research, Merck Research Laboratories, West Point, PA 19486, USA. gideon_rodan@merck.com
Current rheumatology reports (United States) Feb **2003**, 5 (1) p65-74, ISSN 1523-3774 Journal Code: 100888970
Document type: Journal Article
Languages: ENGLISH
Main Citation Owner: NLM
Record type: In Process
The nitrogen-containing bisphosphonates (N-BPs), alendronate and risedronate, are the only pharmacologic agents shown to prevent spine and nonvertebral fractures associated with postmenopausal and glucocorticoid-induced osteoporosis. At the **tissue** level, this is achieved through osteoclast inhibition, which leads to reduced **bone** turnover, increased **bone** mass, and improved **mineralization**. The molecular targets of bisphosphonates (BPs) have recently been identified. This **review** will discuss the mechanism of action of BPs, focusing on alendronate and risedronate, which are the two agents most widely studied. They act on the cholesterol biosynthesis pathway enzyme, farnesyl diphosphate synthase. By inhibiting this enzyme in the osteoclast, they interfere with geranylgeranylation (attachment of the lipid to

regulatory proteins , which causes osteoclast inactivation. This mechanism is responsible for N-BP suppression of osteoclastic **bone** resorption and reduction of **bone** turnover, which leads to fracture prevention.

3/3,AB/2 (Item 2 from file: 155)
DIALOG(R)File 155:MEDLINE(R)

14275658 22342064 PMID: 12454093
Mechanobiology of craniofacial sutures.
Mao J J; et al
Department of Orthodontics MC 841, 801 South Paulina Street, University of Illinois at Chicago, Chicago, IL 60612-7211, USA. jmao2@uic.edu
Journal of dental research (United States) Dec **2002**, 81 (12) p810-6, ISSN 0022-0345 Journal Code: 0354343
Contract/Grant No.: DE13088; DE; NIDCR; DE13964; DE; NIDCR; +
Document type: Journal Article; Review; Review, Tutorial
Languages: ENGLISH
Main Citation Owner: NLM
Record type: Completed
Craniofacial sutures are soft connective **tissue** joints between **mineralized** skull bones. Suture mechanobiology refers to the understanding of how mechanical stimuli modulate sutural growth. This **review** 's hypothesis is that novel mechanical stimuli can effectively modulate sutural growth. Exogenous forces with static, sinusoidal, and square waveforms induce corresponding waveforms of sutural strain. Sutural growth is accelerated upon small doses of oscillatory strain, as few as 600 cycles delivered 10 min/day over 12 days. Interestingly, both oscillatory tensile and compressive strains induce anabolic sutural responses beyond natural growth. Mechanistically, oscillatory strain likely turns on genes and transcription factors that activate cellular machinery via mechanotransduction pathways. Thus, sutural growth is determined by hereditary and mechanical signals via the common pathway of genes. It is concluded that small doses of oscillatory mechanical stimuli have the potential to modulate sutural growth effectively: either accelerating it or initiating net sutural **bone** resorption for various therapeutic objectives.

3/3,AB/3 (Item 3 from file: 155)
DIALOG(R)File 155:MEDLINE(R)

14102388 22131363 PMID: 12136332
Primary synovial chondromatosis and synovial chondrosarcoma: a pictorial **review**.
Wittkop B; Davies A M; Mangham D C; et al
MRI Centre, Royal Orthopaedic Hospital, Birmingham B31 2AP, UK.
European radiology (Germany) Aug **2002**, 12 (8) p2112-9, ISSN 0938-7994 Journal Code: 9114774
Document type: Journal Article
Languages: ENGLISH
Main Citation Owner: NLM
Record type: Completed
This article illustrates the imaging characteristics of primary synovial chondromatosis (PSC) using 20 cases referred to a tertiary orthopaedic oncology centre. Three quarters of patients presented with a large intra-articular soft **tissue** mass and a suspected clinical and radiological diagnosis of malignancy made in the referring centres. Radiographs demonstrated fine cartilaginous **mineralisation** in the soft **tissue** masses in 85% cases and **bone** erosions were shown on MR imaging in 80%. Malignant transformation to chondrosarcoma was proven in 2 cases with longstanding disease. There were no specific MR features to

distinguish these cases with malignant change from PSC alone. Primary synovial chondromatosis should be considered in the diagnosis of the monarticular presentation of an intra articular soft **tissue** mass, particularly in the presence of superficial **bone** erosions and signal voids due to the **mineralisation**.

3/3,AB/4 Item 4 from file: 155
DIALOG(R)File 155:MEDLINE(R)

14032912 22315603 PMID: 12427050
Dietary recommendations and athletic menstrual dysfunction.
Manore Melinda M; et al
Department of Nutrition and Food Management, Oregon State University, Corvallis, Oregon, USA.
Sports medicine (Auckland, N.Z.) (New Zealand) **2002**, 32 (14) p887-901, ISSN 0112-1642 Journal Code: 8412297
Document type: Journal Article
Languages: ENGLISH
Main Citation Owner: NLM
Record type: In Process
Exercise-induced or athletic menstrual dysfunction (amenorrhoea, oligomenorrhoea, anovulation, luteal phase deficiency, delayed menarche) is more common in active women and can significantly affect health and sport performance. Although athletic amenorrhoea represents the most extreme form of menstrual dysfunction, other forms can also result in suppressed estrogen levels and affect **bone** health and fertility. A number of factors, such as energy balance, exercise intensity and training practices, bodyweight and composition, disordered eating behaviours, and physical and emotional stress levels, may contribute to the development of athletic menstrual dysfunction. There also appears to be a high degree of individual variation with respect to the susceptibility of the reproductive axis to exercise and diet-related stresses. The dietary issues of the female athlete with athletic menstrual dysfunction are similar to those of her eumenorrhoeic counterpart. The most common nutrition issues in active women are poor energy intake and/or poor food selection, which can lead to poor intakes of protein, carbohydrate and essential fatty acids. The most common micronutrients to be low are the **bone**-building nutrients, especially calcium, the B vitamins, iron and zinc. If energy drain is the primary contributing factor to athletic menstrual dysfunction, improved energy balance will improve overall nutritional status and may reverse the menstrual dysfunction, thus returning the athlete to normal reproductive function. Because **bone** health can be compromised in female athletes with menstrual dysfunction, intakes of **bone**-building nutrients are especially important. Iron and zinc are typically low in the diets of female athletes if meat products are avoided. Adequate intake of the B vitamins is also important to ensure adequate energy production and the building and repair of muscle **tissue**. This **review** briefly discusses the various factors that may affect athletic menstrual dysfunction and two of the proposed mechanisms: the energy-drain and exercise-intensity hypotheses. Because energy drain can be a primary contributor to athletic menstrual dysfunction, recommendations for energy and the macro- and micronutrients are reviewed. Methods for helping the female athlete to reverse athletic menstrual dysfunction are discussed. The health consequences of trying to restrict energy intake too dramatically while training are also reviewed, as is the importance of screening athletes for disordered eating. Vitamins and **minerals** of greatest concern for the female athlete are addressed and recommendations for intake are given.

3/3,AB/5 Item 5 from file: 155
DIALOG(R)File 155:MEDLINE(R)

13930898 22162675 PMID: 12172035
Angiogenesis and **mineralization** during distraction osteogenesis.
Choi In Ho; Chung Chin Youb; Cho Tae Joon; Yoo Won Joon; et al
Department of Orthopedic Surgery, Seoul National University College of Medicine, Seoul National University Hospital, Seoul, Korea. inhoc@snu.ac.kr
Journal of Korean medical science (Korea South) Aug **2002**, 17 (4) p435-47, ISSN 1011-8934 Journal Code: 8703518
Document type: Journal Article
Languages: ENGLISH
Main Citation Owner: NLM
Record type: In Process
Distraction osteogenesis is currently a standard method of **bone** lengthening. It is a viable method for the treatment of short extremities as well as extensive **bone** defects, because large amounts of **bone** can be regenerated in the distraction gap. Mechanical stimulation by distraction induces biological responses of skeletal regeneration that is accomplished by a cascade of biologic processes that may include differentiation of pluripotential **tissue**, angiogenesis, **mineralization** , and remodeling. There are complex interactions between **bone**-forming osteoblasts and other cells present within the **bone** microenvironment, particularly vascular endothelial cells that may be pivotal members of a complex interactive communication network in **bone**. Regenerate **bone** forms by three modes of ossification, which include intramembranous, enchondral, and transchondroid ossifications, although intramembraneous **bone** formation is the predominant mechanism of ossification. In this **review** we discussed the coupling between angiogenesis and **mineralization**, the biological and mechanical factors affecting them, the cellular and molecular events occurring during distraction osteogenesis, and the emerging modalities to accelerate regenerate **bone** healing and remodeling.

3/3,AB/6 (Item 6 from file: 155)
DIALOG(R)File 155:MEDLINE(R)

13898168 22049901 PMID: 12055335
Body composition of the male and female reference infants.
Fomon Samuel J; Nelson Steven E; et al
Department of Pediatrics, University of Iowa, Iowa City, Iowa 52242-1083, USA. samfomon@aol.com
Annual review of nutrition (United States) **2002**, 22 p1-17, ISSN 0199-9885 Journal Code: 8209988
Document type: Journal Article; Review; Review, Tutorial
Languages: ENGLISH
Main Citation Owner: NLM
Record type: Completed
During infancy, especially early infancy, a substantial proportion of the requirements for energy and specific nutrients are those needed for growth. Knowledge of the body composition of a reference infant (body size and chemical composition at the 50th centile for age) permits an estimate of the growth needs of the infant. In this communication, we **review** efforts from the 1960s to the present at defining the composition of the male and female reference infants. We and others have demonstrated that accumulation of fat is remarkably rapid during the first 4 or 6 months of life. As a percentage of fat-free mass, water decreases throughout infancy whereas protein and **minerals** increase. However, the quantitative nature of these changes remains uncertain. After identifying the areas in which further data are needed, we conclude that the single most important area for further work is determining the relation of "**bone mineral** content" determined by dual energy X-ray absorptiometry to the osseous **mineral** content of the infant.

3/3,AB/7 (Item 7 from file: 155)
DIALOG(R)File 155:MEDLINE(R)

13800032 22261485 PMID: 12373918
[What can we expect of raloxifene in the treatment of postmenopausal osteoporosis- views of a gynecologist]
Co muzeme ocekavat od raloxifenu pri lecbe postmenopauzalni osteoporozy--pohled gynekologa.
Chmel R; Rob L; Strnad P
Gynekologicko-porodnicka klinika, UK, 2. LF a FN v Motole, Praha. Chmel.Roman@seznam.cz
Ceska gynekologie / Ceska lekarska spolecnost J. Ev. Purkyne (Czech Republic) Jul **2002**, 67 (4) p187-91, ISSN 1210-7832
Journal Code: 9423768
Document type: Journal Article ; English Abstract
Languages: CZECH
Main Citation Owner: NLM
Record type: Completed
OBJECTIVE: Evaluation of positive properties and side effects of raloxifene treatment with respect to its potential use as agent to improve women's health and quality of life in postmenopausal years. DESIGN: A **review** article. SETTING: Obstetrics and Gynaecology Department, Charles University 2nd Medical Faculty and Teaching Hospital Motol, Prague. SUBJECT: Estrogen use may protect against osteoporosis and cardiovascular disease, but may increase the risk of breast cancer in long-term treated women and also may increase the risk of irregular uterine bleeding (in combination with gestagen in non-hysterectomized women) in perimenopause and postmenopause. Drugs with **tissue**-specific estrogenic effects are termed selective estrogen receptor modulators (SERM). Tamoxifen is the first SERM successfully used in the prevention and treatment of breast cancer. Another SERM raloxifene is widely used in the prevention and treatment of postmenopausal osteoporosis, especially in women without climacteric complaints. Therapy with raloxifene increases **bone mineral** density, lowers serum concentrations of total and low-density lipoprotein cholesterol, and does not stimulate endometrium and breast. Evaluation of another potential positive effects (reducing size of uterine leiomyomas, etc.) warrants further investigation. CONCLUSION: Raloxifene can be used in postmenopausal women free of climacteric symptoms for the prevention and treatment of postmenopausal osteoporosis with no increased risk of thrombosis and with the advantage of positive side effects during the treatment.

3/3,AB/8 (Item 8 from file: 155)
DIALOG(R)File 155:MEDLINE(R)

13780142 22202060 PMID: 12213663
Anorexia nervosa in female adolescents: endocrine and **bone mineral** density disturbances.
Munoz M T; Argente J
Division of Pediatric Endocrinology, Hospital Universitario Infantil Nino Jesus, Avda Menendez Pelayo 65, E-28009 Madrid, Spain.
European journal of endocrinology / European Federation of Endocrine Societies (England) Sep **2002**, 147 (3) p275-86, ISSN 0804-4643
Journal Code: 9423848
Document type: Journal Article; Review; Review, Tutorial
Languages: ENGLISH
Main Citation Owner: NLM
Record type: Completed
Anorexia nervosa (AN) is a chronic childhood psychiatric illness that

involves a reduction in caloric intake, loss of weight and amenorrhea, either primary or secondary. The diagnostic criteria for AN have been established by the American Psychiatric Association. The prevalence of this disease amongst adolescents and young adults is between 0.5 and 1% and the incidence of new cases per year is approximately 5-10/100,000 between 15 and 19 years of age. A number of endocrine and metabolic disturbances have been described in patients with AN including amenorrhea-oligomenorrhea, delayed puberty, hypothyroidism, hypercortisolism, IGF-I deficiency, electrolyte abnormalities, hypoglycemia and hypophosphatemia, among others. In addition to prolonged amenorrhea, osteopenia and osteoporosis are the most frequent complications leading to clinically relevant fractures and increased fracture risk throughout life. Patients exhibit an alteration in the hypothalamic-pituitary-gonadal axis, which is responsible for the menstrual disorders. The increase in gonadotropin secretion that can be observed after ponderal recuperation suggests that malnutrition could be the most important mechanism involved in the decrease in gonadotropin secretion. The loss of fat **tissue** as a consequence of nutrient restriction has been associated with hypoleptinemia and abnormal secretion of peptides implicated in food control (neuropeptide Y, melanocortins and corticotropin-releasing factor, among others). A **review** of the endocrine abnormalities, disturbances in neurotransmitters, as well as a detailed analysis of **bone** markers and **bone mineral** density in patients with AN is described.

3/3/AB/9 (Item 9 from file: 155)
DIALOG(R)File 155:MEDLINE(R)

13548044 21914705 PMID: 11916540

Role of Ca(2+) and vitamin D in the prevention and treatment of osteoporosis.

Rodriguez-Martinez M A; Garcia-Cohen E C

Unidad de Ensayos Clinicos y Area de Investigacion Farmacologica, Servicio de Farmacologia Clinica, Hospital Universitario Clinica Puerta de Hierro de Madrid, C/ San Martin de Porres 4, 28035 Madrid, Spain. mariangeles.rodriguez@uam.es

Pharmacology & therapeutics (England) Jan **2002**, 93 (1) p37-49, ISSN 0163-7258 Journal Code: 7905840

Document type: Journal Article; Review; Review, Tutorial

Languages: ENGLISH

Main Citation Owner: NLM

Record type: Completed

Osteoporosis is defined as a progressive systemic skeletal disease characterised by low **bone** mass and microarchitectural deterioration of **bone tissue**, with a consequent increase in **bone** fragility and susceptibility to fracture. The clinical relevance of osteoporosis derives from the fractures that it produces. More than one-third of the adult women will suffer one or more osteoporotic fractures in their lifetime. The lifetime risk in men is approximately one-half that in women. The decrease of the **bone mineral** density is the most important cause of risk fracture. Among other factors, Ca(2+) and vitamin D deficiencies are important risk factors for a decrease in **bone mineral** density, consequently inducing osteoporosis. The high prevalence of vitamin D deficiency in healthy elderly people living mainly in southern European countries increase the risk of osteoporotic fractures in these populations above those anticipated for the general elderly population of the European community. In addition, the ageing of the European population will double the number of osteoporotic fractures over the next 50 years, unless adequate preventative measures are undertaken. The efficacy and safety of Ca(2+) and vitamin D supplements at preventing **bone** loss and reducing the risk of hip and other fractures have been assessed in different clinical trials, which are extensively discussed in this **review**.

3/3,AB/10 (Item 10 from file: 155)
DIALOG(R)File 155:MEDLINE(R)

13093258 21934894 PMID: 11937868
Properties of osteoconductive biomaterials: calcium phosphates.

LeGeros Racquel Zapanta
Department of Biomaterials and Biomimetics, New York University College of Dentistry, New York 10010, USA.

Clinical orthopaedics and related research (United States) Feb **2002**, (395) p81-98, ISSN 0009-921X Journal Code: 0075674
Contract/Grant No.: DE 04123; DE; NIDCR; DE 12388; DE; NIDCR; DE07223; DE; NIDCR; S07RR07626; RR; NCRR
Document type: Journal Article; Review; Review, Academic
Languages: ENGLISH
Main Citation Owner: NLM
Record type: Completed

Bone is formed by a series of complex events involving the **mineralization** of extracellular matrix proteins rigidly orchestrated by cells with specific functions of maintaining the integrity of the **bone**. **Bone**, similar to other calcified tissues, is an intimate composite of the organic (collagen and noncollagenous proteins) and inorganic or **mineral** phases. The **bone mineral** idealized as calcium hydroxyapatite, Ca10 (PO4)(6)(OH)2, is a carbonatehydroxyapatite, approximated by the formula: (Ca,X)(10)(PO4,HPO4,CO3)(6)(OH,Y)2, where X are cations (magnesium, sodium, strontium ions) that can substitute for the calcium ions, and Y are anions (chloride or fluoride ions) that can substitute for the hydroxyl group. The current author presents a brief **review** of CaP biomaterials that now are used as grafts for **bone** repair, augmentation, or substitution. Commercially-available CaP biomaterials differ in origin (natural or synthetic), composition (hydroxyapatite, beta-tricalcium phosphate, and biphasic CaP), or physical forms (particulates, blocks, cements, coatings on metal implants, composites with polymers), and in physicochemical properties. CaP biomaterials have outstanding properties: similarity in composition to **bone mineral**; bioactivity (ability to form **bone** apatitelike material or carbonate hydroxyapatite on their surfaces), ability to promote cellular function and expression leading to formation of a uniquely strong **bone**-CaP biomaterial interface; and osteoconductivity (ability to provide the appropriate scaffold or template for **bone** formation). In addition, CaP biomaterials with appropriate three-dimensional geometry are able to bind and concentrate endogenous **bone** morphogenetic proteins in circulation, and may become osteoinductive (capable of osteogenesis), and can be effective carriers of **bone** cell seeds. Therefore, CaP biomaterials potentially are useful in **tissue** engineering for regeneration of hard tissues.

3/3,AB/11 (Item 11 from file: 155)
DIALOG(R)File 155:MEDLINE(R)

12995546 21572092 PMID: 11716022
Biology of bone and how it orchestrates the form and function of the skeleton.

Sommerfeldt D W; Rubin C T
Center for Biotechnology, State University of New York, Stony Brook, USA. Clinton.Rubin@sunysb.edu

European spine journal : official publication of the European Spine Society, the European Spinal Deformity Society, and the European Section of the Cervical Spine Research Society (Germany) Oct **2001**, 10 Suppl 2 pS86-95, ISSN 0940-6719 Journal Code: 9301980

Contract/Grant No.: AR39278; AR; NIAMS; AR41111; AR; NIAMS; AR41040; AR; NIAMS; AR43457; AR; NIAMS
Document type: Journal Article; Review; Review, Tutorial
Languages: ENGLISH
Main Citation Owner: NLM
Record type: Completed

The principal role of the skeleton is to provide structural support for the body. While the skeleton also serves as the body's **mineral** reservoir, the **mineralized** structure is the very basis of posture, opposes muscular contraction resulting in motion, withstands functional load bearing, and protects internal organs. Although the mass and morphology of the skeleton is defined, to some extent, by genetic determinants, it is the **tissue** 's ability to remodel--the local resorption and formation of **bone**--which is responsible for achieving this intricate balance between competing responsibilities. The aim of this **review** is to address **bone** 's form-function relationship, beginning with extensive research in the musculoskeletal disciplines, and focusing on several recent cellular and molecular discoveries which help understand the complex interdependence of **bone** cells, growth factors, physical stimuli, metabolic demands, and structural responsibilities. With a clinical and spine oriented audience in mind, the principles of **bone** cell and molecular biology and physiology are presented, and an attempt has been made to incorporate epidemiologic data and therapeutic implications. **Bone** research remains interdisciplinary by nature, and a deeper understanding of **bone** biology will ultimately lead to advances in the treatment of diseases and injuries to **bone** itself.

3/3,AB/12 (Item 12 from file: 155)
DIALOG(R)File 155:MEDLINE(R)

12951314 21869876 PMID: 11880731
Marfan syndrome: orthopedic and genetic **review**.
Giampietro Philip F; Raggio Cathleen; Davis Jessica G
Division of Genetics, Department of Pediatrics, Weill Medical College of Cornell University, USA. giampiep@mfldclin.edu
Current opinion in pediatrics (United States) Feb **2002**, 14 (1) p35-41, ISSN 1040-8703 Journal Code: 9000850
Contract/Grant No.: M01RR00047; RR; NCRR; M01RR06020; RR; NCRR
Document type: Journal Article; Review; Review, Tutorial
Languages: ENGLISH
Main Citation Owner: NLM
Record type: Completed

Marfan syndrome is an autosomal dominant disorder of connective **tissue** that affects the cardiac, eye, and skeletal systems. More than 135 mutations have been identified in the fibrillin-1 gene, localized on chromosome 15q(21.1) and responsible for the clinical manifestations of Marfan syndrome. The major orthopedic manifestations of Marfan syndrome include scoliosis, chest wall deformity, dural ectasia, joint hypermobility, and acetabular protrusion. In addition, decreased **bone mineral** density has been reported in patients with Marfan syndrome. This **review** summarizes recent developments in the genetic and orthopedic aspects of Marfan syndrome. Increased practitioner awareness of the clinical features associated with Marfan syndrome may facilitate earlier diagnosis and optimize patient treatment.

3/3,AB/13 (Item 13 from file: 155)
DIALOG(R)File 155:MEDLINE(R)

12763955 21451021 PMID: 11566262
Regulated gene expression dictates enamel structure and tooth function.

Paine M L; White S N; Luo W; Fong H; Sarikaya M; Snead M L
University of Southern California, Center for Craniofacial Molecular Biology, Los Angeles, CA 90033, USA. paine hsc.usc.edu
Matrix biology : journal of the International Society for Matrix Biology (Germany) Sep **2001**, 20 (5-6) p273-92, ISSN 0945-053X
Journal Code: 9432592
Contract Grant No.: DE-02848; DE; NIDCR; DE-06988; DE; NIDCR; DE-11704; DE; NIDCR; DE-12350; DE; NIDCR; DE-12420; DE; NIDCR; DE-13045; DE; NIDCR; DE-13404; DE; NIDCR
Document type: Journal Article; Review; Review, Tutorial
Languages: ENGLISH
Main Citation Owner: NLM
Record type: Completed
Enamel is a complex bioceramic **tissue**. In its final form, enamel is a reflection of the unique molecular and cellular activities occurring during organogenesis. From the ectodermal origins of ameloblasts, their gene activity and protein expression profiles exist for the sole purpose of producing a **mineralized** shell, almost entirely devoid of protein, deposited over the '**bone**-like' dentine. The interface between enamel and dentine is referred to as the dentine enamel junction and it is also unique in its biology. This **review** article is narrow in its scope. We restrict our **review** to selected advances in our understanding of the genetic, molecular and structural aspects of enamel biology. We present a model of enamel formation that relates gene expression to the assembly of an extracellular protein matrix that in turn controls the structural hierarchy and mechanical aspects of enamel and the tooth organ.

3/3,AB/14 (Item 14 from file: 155)
DIALOG(R)File 155:MEDLINE(R)

12589895 21535378 PMID: 11680687
The role of ERG (ets related gene) in cartilage development.
Iwamoto M; Higuchi Y; Enomoto-Iwamoto M; Kurisu K; Koyama E; Yeh H; Rosenbloom J; Pacifici M
Department of Oral Anatomy and Developmental Biology, Osaka University Faculty of Dentistry, Osaka, Japan. mal@dent.osaka-u.ac.jp
Osteoarthritis and cartilage / OARS, Osteoarthritis Research Society (England) **2001**, 9 Suppl A pS41-7, ISSN 1063-4584 Journal Code: 9305697
Document type: Journal Article
Languages: ENGLISH
Main Citation Owner: NLM
Record type: Completed
OBJECTIVE: Based on function and developmental fate, cartilage **tissue** can be broadly classified into two types: transient (embryonic or growth-plate) cartilage and permanent cartilage. Chondrocytes in transient cartilage undergo terminal differentiation into hypertrophic cells, induce cartilage-matrix **mineralization** , and eventually disappear and are replaced by **bone**. On the other hand, chondrocytes in permanent cartilage do not differentiate further, do not become hypertrophic, and persist throughout life at specific sites, including joints and tracheal rings. While many studies have described differences in structure, matrix composition and biological characteristics between permanent and transient cartilage, it is poorly understood how the fates of permanent and transient cartilage are determined. Previous studies demonstrated that chondrocytes isolated from permanent cartilage have the potential to express markers of the mature hypertrophic phenotype once grown in culture, suggesting that cell hypertrophy is an intrinsic property of all chondrocytes and must be actively silenced in permanent cartilage in vivo. These silencing mechanisms, however, are largely unknown. In this paper, we first **review** nature of chondrocytes in transient and permanent cartilages and then report the cloning and characterization of a

novel variant of ets transcription factor chERG, hereafter called C-1-1, which might be involved in regulation of permanent cartilage development. DESIGN: For cloning of a novel variant of chERG C-1-1, we isolated RNA from the cartilaginous femur or tibiotarsus of Day 17 chick embryos and processed it for reverse transcription-polymerase chain reaction (RT-PCR) with the primers from sequences upstream and downstream of the 81 and 72 bp segments alternatively spliced in mammals. For investigation of function of chERG and C-1-1, we over-expressed chERG or C-1-1 in cultured chick chondrocytes or the developing limb of chick embryo using a retrovirus (RCAS) system, and examined the phenotype changes in the infected chondrocytes or the infected limb elements. RESULTS: C-1-1 is an alternative and novel variant lacking the 27 amino acids segment of chERG that has been reported previously. C-1-1 is preferentially expressed in developing articular cartilage, whereas chERG is preferentially expressed in growth plate cartilage. Growth of articular chondrocytes in culture was accompanied by decreasing C-1-1 expression after several passages, while expression of hypertrophic markers increased. Expression of C-1-1 in cultured chondrocytes inhibited cell hypertrophy, alkaline phosphatase activity, and cartilage matrix **mineralization** . In contrast, over-expression of chERG promoted chondrocyte maturation and **mineralization** . CONCLUSION: Our data demonstrate for the first time that chERG and C-1-1 play distinct roles in skeletogenesis and may have crucial roles in the development and function of transient and permanent cartilages.

3/3,AB/15 (Item 15 from file: 155)
DIALOG(R)File 155:MEDLINE(R)

12556944 21461709 PMID: 11577963
A bioengineered implant for a predetermined **bone** cellular response to loading forces. A literature **review** and case report.
Misch C E; Bidez M W; Sharawy M
University of Michigan School of Dentistry, Department of Periodontics, Ann Arbor, USA. info@misch.com
Journal of periodontology (United States) Sep **2001**, 72 (9) p1276-86, ISSN 0022-3492 Journal Code: 8000345
Document type: Journal Article; Review; Review, Tutorial
Languages: ENGLISH
Main Citation Owner: NLM
Record type: Completed
The presence of fibrous **tissue** has long been known to decrease the long-term survival of a root-form implant. Excessive loads on an osseointegrated implant may result in mobility of the supporting device, and excessive loads may also fracture an implant component or body. Although several conditions may cause crestal **bone** loss, one of these may be prosthetic overload. Excessive loads on the **bone** cause strain conditions to increase. These microstrains on the **bone** may affect the **bone** remodeling rate in a direct relationship. When strain conditions to the interfacial **bone** are in the mild overload zone, an increased **bone** remodeling response occurs, which results in a reactive woven **bone** formation that is less **mineralized** and weaker. Greater stresses may cause the interfacial strain to reach the pathologic overload zone and may cause microfracture of the **bone**, fibrous **tissue** formation, and/or **bone** resorption. Recent reports suggest that the **bone** remodeling rate next to an implant may be used to evaluate biomechanical conditions and their influence on the implant-to-**bone** interface. These include a number of factors, such as loading conditions, implant body surface conditions, and implant design. For a given load condition, the implant design is one of the primary factors that determine the resultant strain at the interface. A predetermined goal was established to bioengineer a dental implant to load the **bone** at the interface in a predetermined stress-strain relationship, in order to maintain lamellar

bone at the interface. A case report is presented of 2 bioengineered implants loaded for 1 year, which demonstrates that the **bone** was primarily lamellar in structure, the **bone** turnover rate was less than 5 microns day, and was the same as the **bone** away from the interface. These findings corroborate those observed in a prior animal study reported with the same implant design. Although the number of implants evaluated in those 2 reports is few, they support a predetermined histological outcome.

3/3,AB/16 (Item 16 from file: 155)
DIALOG(R)File 155:MEDLINE(R)

12509351 21065872 PMID: 11137570
Bioactive fatty acids: role in **bone** biology and **bone** cell function.
Watkins B A; Lippman H E; Le Bouteiller L; Li Y; Seifert M F
Department of Food Science, Lipid Chemistry and Molecular Biology Laboratory, Purdue University, 47907, West Lafayette, IN, USA
Progress in lipid research (England) Jan-Mar **2001**, 40 (1-2) p125-48, ISSN 0163-7827 Journal Code: 7900832
Document type: Journal Article; Review; Review, Academic
Languages: ENGLISH
Main Citation Owner: NLM
Record type: Completed
Bone is a unique **tissue** providing support, movement, and **mineral** balance for the body. **Bone** growth is achieved in the young by a process called modeling, and maintained during adulthood by a process termed remodeling. Three types of cells are responsible for the formation of cartilage and **bone**; the chondrocyte, osteoblast, and osteoclast. These cells are under the influence of a plethora of regulatory molecules, which govern their action to provide an individual optimal **bone** mass. Interruption of this homeostatic machinery, especially in the elderly, often results in a loss of **bone** mass (osteoporosis) or cartilage damage (rheumatoid arthritis). Many pharmacolcgical agents have been made available in an effort to prevent or alleviate these pathologies, however, one vector often overlooked is the diet. This **review** focuses on the relationship between dietary polyunsaturated fatty acids and **bone** biology, both in vivo and in vitro.

3/3,AB/17 (Item 17 from file: 155)
DIALOG(R)File 155:MEDLINE(R)

12518338 21353652 PMID: 11460594
Diabetes mellitus a risk for osteoporosis?
Leidig-Bruckner G; Ziegler R
Department of Internal Medicine I, Endocrinology and Metabolism, University of Heidelberg, Germany.
Experimental and clinical endocrinology & diabetes : official journal, German Society of Endocrinology and German Diabetes Association (Germany) **2001**, 109 Suppl 2 pS493-514, ISSN 0947-7349 Journal Code: 9505926
Document type: Journal Article; Review; Review, Tutorial
Languages: ENGLISH
Main Citation Owner: NLM
Record type: Completed
Localized lesions at the foot skeleton are a serious and well recognized complication of diabetes mellitus which may impair the clinical outcome of the patients remarkably. In contrast, the presence of a generalized **bone** disease or osteoporosis related to diabetes mellitus is less acknowledged and its clinical relevance is less obvious. This paper is a clinically focused **review** of the literature on osteoporosis related

to diabetes mellitus. Due to the different pathogenesis of diabetes mellitus type 1 and type 2 it is not surprising that there is no uniform entity of diabetic osteopathy. The majority of clinical studies in subjects with diabetes mellitus type 1 showed a moderately decreased **bone** mass at the forearm, while **bone** mass at the femur or lumbar spine was either decreased or not different from non-diabetic controls. In patients with diabetes mellitus type 2 the risk of osteopenia is not as clear as in type 1 diabetes. **Bone mineral** density at the forearm in patients with type 2 diabetes mellitus was decreased, unchanged or even increased in comparison to controls, while **bone mineral** density at the vertebrae or femoral neck was either not significantly different or increased, but rarely decreased. The underlying mechanisms triggering changes in **bone** mass in patients with diabetes mellitus type 1 and type 2 are not well known. In most studies there was no consistent relationship between the metabolic control of diabetes and **bone mineral** density. Biochemical parameters of the calcium and **bone** metabolism showed no clear relationship to the **bone mineral** density measurements. From few **bone** histology studies in humans and experimental studies there is evidence that a decreased **bone** formation is one major mechanism leading to reduced **bone** mass in diabetics. Microangiopathy at the **bone tissue** was also discussed as a possible reason for diabetic osteopenia. It was shown that insulin and insulin like growth factors (IGF-1, IGF-2) have an influence on **bone** metabolism itself and other growth factors, cytokines and hormones may determine changes in diabetic **bone** metabolism. Recent findings suggest that leptin is involved in the regulation of osteoblast function and **bone** mass, which is of special interest in diabetes mellitus type 2. The clinical relevance of osteoporosis or osteopenia is determined by the increased risk for insufficiency fractures. Few studies found an increased fracture risk, especially in older women with type 1 diabetes mellitus, while others did not show an increased risk for fractures or even found a decreased rate of fractures in women with diabetes mellitus type 2. There is a need for further longitudinal studies, including the incidence and risk factors for osteoporotic fractures. In clinical routine the extent of diagnostic and therapeutic activities in patients with type 1 or type 2 diabetes mellitus in respect to generalized **bone** disease or diabetic osteopenia should be based on individual conditions and risk profile for osteoporosis.

3/3,AB/18 (Item 18 from file: 155)
DIALOG(R)File 155:MEDLINE(R)

11344123 21410476 PMID: 11519708
Efficacy of porous bovine **bone mineral** in various types of osseous deficiencies: clinical observations and literature **review**.
Artzi Z; Nemcovsky C E; Tal H
Department of Periodontology, The Maurice and Gabriela Goldschleger School of Dental Medicine, Tel Aviv University, Israel.
International journal of periodontics & restorative dentistry (United States) Aug **2001**, 21 (4) p395-405, ISSN 0198-7569
Journal Code: 8200894
Document type: Journal Article
Languages: ENGLISH
Main Citation Owner: NLM
Record type: Completed
Recent developments in osseous regenerative techniques have increased the demand for **bone**-substitute grafting materials. Porous deproteinized bovine **bone mineral** (PBBM), a biocompatible xenograft, has been used in different osseous deficiencies prior to or in conjunction with the placement of titanium implants. The different PBBM applications in fresh extraction sites, anatomic defects, and subantral floor elevation techniques are described. The use of an occlusive barrier membrane to

regenerate **bone** via guided **tissue** regeneration principles was determined for each patient by clinical parameters. PBBM was well amalgamated and incorporated with the augmented hard **tissue**, but the transition between preexisting **bone** and the newly regenerated **bone** like **tissue** was distinguishable by clinical examination even after 10 months. Grafted material was also identified using follow-up radiographs. In the presented cases, PBBM showed clinically satisfactory results as a biocompatible filler in **bone** augmentation procedures.

3/3,AB/19 (Item 19 from file: 155)
DIALOG(R)File 155:MEDLINE(R)

11335599 21394411 PMID: 11503140
Biochemical basis of the pharmacologic action of chondroitin sulfates on the osteoarticular system.
Bali J P; Cousse H; Neuzil E
Faculty of Pharmacy, University Montpellier I, France. jbali ww3.pharma.univ-montp1.fr
Seminars in arthritis and rheumatism (United States) Aug **2001**, 31 (1) p58-68, ISSN 0049-0172 Journal Code: 1306053
Document type: Journal Article; Review; Review, Tutorial
Languages: ENGLISH
Main Citation Owner: NLM
Record type: Completed
BACKGROUND: Chondroitin sulfates (CS) are involved in articular metabolism and could be used as therapeutic agents in degenerative articular diseases. OBJECTIVES: To **review** the published reports describing both the metabolism of glycosaminoglycans (GAG) and their involvement in osteoarticular pathophysiology. METHODS: MEDLINE search for relevant articles and **review** of cited references. RESULTS: 1) CS are formed of disaccharide units; sulfated galactosamine residues in position 4 or 6 are found in various ratios, depending on the age and the type of **tissue** . Binding to the core protein through N- and O-linkages leads to aggregates of monomers with high molecular weights. The proteoglycan aggregate exhibits viscoelastic and hydration properties and an ability to interact with the surrounding **tissue** through electric charges leading to protection of the cartilaginous tissues. 2) CS are synthesized both in chondrocytes and in **bone** cells by the action of specific glycosyl-transferases; their catabolism occurs in the matrix and involves numerous matrix (metalloproteinases) and lysosomal enzymes. 3) CS are inhibitors of extracellular proteases involved in the metabolism of connective tissues. In addition to their anti-inflammatory effects, CS in vitro stimulate proteoglycan production by chondrocytes; they also inhibit cartilage cytokine production and induce apoptosis of articular chondrocytes. CS increase the intrinsic viscosity of the synovial liquid. 4) In vivo in experimental arthritis, the number and severity of articular symptoms decreases after CS administration. In bones, CS accelerate the **mineralization** process and **bone** repair. CONCLUSIONS: All these data suggest that CS play a role in articular and **bone** metabolism by controlling cartilaginous matrix integrity and **bone** **mineralization**. Copyright 2001 by W.B. Saunders Company

3/3,AB/20 (Item 20 from file: 155)
DIALOG(R)File 155:MEDLINE(R)

11284667 21321322 PMID: 11428176
Tibolone and its effects on **bone**: a **review**.
Berning B; Bennink H J; Fauser B C
Department of Obstetrics and Gynecology, Leyenburg Hospital, The Hague.
Climacteric : the journal of the International Menopause Society (United

States Jun 2001, 4 (2) p120-36, ISSN 1369-7137
Journal Code: 9810959
Document type: Journal Article; Review; Review, Tutorial
Languages: ENGLISH
Main Citation Owner: NLM
Record type: Completed
This **review** examines the evidence for the effects of tibolone on **bone** . Tibolone is a synthetic steroid with a mixed estrogenic-progestogenic-androgenic hormonal profile. Data suggest a complex receptor-mediated as well as metabolic regulation of the activity of tibolone at target **tissue** level. It has been shown that tibolone can prevent axial and appendicular **bone** loss induced by ovariectomy and/or a low-calcium diet in young and mature rats. In addition, tibolone increases trabecular and cortical **bone mineral** density in rats with established osteopenia. In the rat, treatment with tibolone results in an increased strength of the femoral neck and of the vertebral body, similar to that found with estrogens. The protective effect on **bone** can be blocked by antiestrogens, indicating that the effect is estrogen receptor-mediated. Clinical trials have shown that loss of **bone** in the spine and proximal hip can be prevented with tibolone 2.5 mg/day in early- and late-postmenopausal women. In addition, a dose of 1.25 mg/day seems also to be effective, especially in late-postmenopausal women. In women with established osteoporosis, **bone** density of the axial and appendicular skeleton increases with tibolone. In comparative studies, tibolone 2.5 mg/day seems to be as effective as conventional hormone replacement therapy regimens. There are no direct comparative studies between tibolone and bisphosphonates or raloxifene. Furthermore, to establish the efficacy of tibolone for prevention of osteoporotic fractures, studies of the magnitude of reduction in fracture risk remain to be conducted. Finally, tibolone seems to be effective in preserving **bone** density in patients treated with gonadotropin-releasing hormone agonist.

3/3,AB/21 (Item 21 from file: 155)
DIALOG(R)File 155:MEDLINE(R)

11278599 21319554 PMID: 11426002

Intramedullary osteosclerosis: imaging features in nine patients.

Chanchairujira K; Chung C B; Lai Y M; Haghighi P; Resnick D
Departments of Radiology, Veterans Affairs Medical Center, University of California-San Diego, 3350 La Jolla Village Dr, San Diego, CA 92161, USA.
Radiology (United States) Jul **2001**, 220 (1) p225-30, ISSN 0033-8419 Journal Code: 0401260
Document type: Journal Article
Languages: ENGLISH
Main Citation Owner: NLM
Record type: Completed
PURPOSE: To determine the conventional radiographic, computed tomographic (CT), magnetic resonance (MR) imaging, scintigraphic, and histologic features of intramedullary osteosclerosis and to **review** the clinical features. MATERIALS AND METHODS: Nine female patients with leg pain and imaging features indicative of intramedullary sclerosis were seen during a 25-year period. None of the patients had a history of trauma or infection, familial **bone** disease, or related abnormal laboratory findings. Imaging studies included radiography (n = 9), CT (n = 4), MR imaging (n = 5), and skeletal scintigraphy (n = 5). Histologic correlation was available in five patients. RESULTS: Sixteen **bone** lesions (midtibia, n = 14; distal fibula, n = 1; and proximal femur, n = 1) were evident. Both lower extremities were involved in seven patients, and a single extremity was involved in two. Intramedullary sclerosis was present, as was cortical thickening, mainly in the diaphysis of the long bones, without extensive periosteal reaction or soft-**tissue** involvement. Findings at

bone scintigraphy were positive in all lesions. Histologic analysis showed nonspecific changes of markedly sclerotic **bone** with a variable degree of **mineralization** and maturity. CONCLUSION: Intramedullary osteosclerosis is a distinct disorder that typically affects the diaphysis of one or both tibiae in women. Characteristic imaging findings, when coupled with clinical information, allow precise diagnosis.

3/3,AB/22 (Item 22 from file: 155)
DIALOG(R)File 155:MEDLINE(R)

11180499 21194865 PMID: 11298188
Calcitonin and calcitonin receptors: **bone** and beyond.
Pondel M
Department of Cellular Pathology, St. George's Hospital Medical School, Cranmer Terrace, London SW17 0RE, UK. mpondel@sghms.ac.uk
International journal of experimental pathology (England) Dec **2000**, 81 (6) p405-22, ISSN 0959-9673 Journal Code: 9014042
Document type: Journal Article; Review; Review Literature
Languages: ENGLISH
Main Citation Owner: NLM
Record type: Completed
Calcitonin (CT), a 32 amino acid peptide hormone produced primarily by the thyroid, and its receptor (CTR) are well known for their ability to regulate osteoclast mediated **bone** resorption and enhance Ca2+ excretion by the kidney. However, recent studies now suggest that CT and CTRs may play an important role in a variety of processes as wide ranging as embryonic/foetal development and sperm function/physiology. In this **review** article, CT and CTR gene transcription, signal transduction and function are addressed. The effects of CT on the physiology of a variety of organ systems are discussed and the relationship between polymorphisms in the CTR gene and **bone mineral** density (BMD)/osteoporosis is examined. Recent studies demonstrating the ability of receptor activity modifying proteins (RAMPs) to post-translationally modify the calcitonin receptor-like receptor (CRLR) are detailed and studies employing transgenic mouse technology to determine the temporal and **tissue** specific transcriptional activity of the CTR gene in vivo are discussed.

3/3,AB/23 (Item 23 from file: 155)
DIALOG(R)File 155:MEDLINE(R)

11166302 21212581 PMID: 11315987
Changes in **bone** density during childhood and adolescence: an approach based on **bone**'s biological organization.
Rauch F; Schoenau E
Children's Hospital, University of Cologne, Germany.
Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research (United States) Apr **2001**, 16 (4) p597-604, ISSN 0884-0431 Journal Code: 8610640
Document type: Journal Article; Review; Review, Tutorial
Languages: ENGLISH
Main Citation Owner: NLM
Record type: Completed
Bone densitometry has great potential to improve our understanding of **bone** development. However, densitometric data in children rarely are interpreted in light of the biological processes they reflect. To strengthen the link between **bone** densitometry and the physiology of **bone** development, we **review** the literature on physiological mechanisms and structural changes determining **bone mineral** density (BMD). BMD (defined as mass of **mineral** per unit volume) is

analyzed in three levels: in **bone** material BMD material , in a **bone**'s trabecular and cortical **tissue** compartments BMD compartment , and in the entire **bone** BMD total . BMD material of the femoral midshaft cortex decreases after birth to a nadir in the first year of life and thereafter increases. In iliac trabecular **bone** , BMD material also increases from infancy to adulthood, reflecting the decrease in **bone** turnover. BMD material cannot be determined with current noninvasive techniques because of insufficient spatial resolution. BM compartment of the femoral midshaft cortex decreases in the first months after birth followed by a rapid increase during the next 2 years and slower changes thereafter, reflecting changes in both relative **bone** volume and BMD(material). Trabecular BMD(compartment) increases in vertebral bodies but not at the distal radius. Quantitative computed tomography (QCT) allows for the determination of both trabecular and cortical BMD(compartment), whereas projectional techniques such as dual-energy X-ray absorptiometry (DXA) can be used only to assess cortical BMD(compartment) of long **bone** diaphyses. BMD(total) of long bones decreases by about 30% in the first months after birth, reflecting a redistribution of **bone tissue** from the endocortical to the periosteal surface. In children of school age and in adolescents, changes in BMD(total) are site-specific. There is a marked rise in BMD(total) at locations where relative cortical area increases (metacarpal bones, phalanges, and forearm), but little change at the femoral neck and midshaft. BMD(total) can be measured by QCT at any site of the skeleton, regardless of **bone** shape. DXA allows the estimation of BMD(total) at skeletal sites, which have an approximately circular cross-section. The system presented here may help to interpret densitometric results in growing subjects on a physiological basis.

3/3,AB/24 (Item 24 from file: 155)
DIALOG(R)File 155:MEDLINE(R)

11089287 21087067 PMID: 11218500
Tools for **tissue** engineering of **mineralized** oral structures.
Dard M; Sewing A; Meyer J; Verrier S; Roessler S; Scharnweber D
Merck Biomaterial, Frankfurter Strasse 250, 64271 Darmstadt, Germany.
Clinical oral investigations (Germany) Jun **2000**, 4 (2) p126-9,
ISSN 1432-6981 Journal Code: 9707115
Document type: Journal Article; Review; Review, Tutorial
Languages: ENGLISH
Main Citation Owner: NLM
Record type: Completed
This paper presents a short **review** of three groups of tools which can be or are used for the **tissue** engineering of **mineralized** oral structures: growth factor delivery systems (GFDS) and surface bioactivation with covalent bound peptides or with nanomechanically linked proteins. According to the reported personal experience of the authors, GFDS have to face the following challenging issue before being used routinely in dentistry, e.g., as a tool for reparative dentinogenesis or **bone** healing: adaptation of the GFDS design to the **tissue** where it will be implanted in order to deliver the right dose of growth factor (GF) at the right time. The bioactivation of surfaces, for example of dental implants, with covalent bound peptides or nanomechanically linked proteins represents a second innovative way to improve dental health in the future. Here we report on the experimental use of cyclic RGD peptides grafted on polymethylmethacrylate to improve osteoblast adhesion. Furthermore, we show the potential advantage of immobilizing and incorporating collagen I on titanium implant surfaces. These techniques or a combination of them will help to create improvements, for example, of dental implants in the near future. They will also help to promote **bone** and dentin regeneration.

3/3,AB/25 (Item 25 from file: 155)
DIALOG(R)File 155:MEDLINE(R)

11028069 21022733 PMID: 11141956
[Celiac disease today: **review** of the growing knowledge]
A coeliakia napjainkban: a bovulo ismeretek attekintese.
Juhasz M; Zagoni T; Toth M; Tulassay Z
Altalanos Orvostudomanyi Kar, II. Belgyogyaszati Klinika, Semmelweis Egyetem, Budapest.
Orvosi hetilap (Hungary) Nov 26 **2000**, 141 (48) p2583-93, ISSN 0030-6002 Journal Code: 0376412
Document type: Journal Article; Review; Review, Tutorial ; English Abstract
Languages: HUNGARIAN
Main Citation Owner: NLM
Record type: Completed
Coeliac disease is the most common disorder with malabsorption of the small intestine, caused by the gluten fraction of cereals in genetically predisposed individuals. T-cell mediated autoimmune processes are initiated by gluten exposure, leading to both intestinal and extraintestinal manifestations, therefore coeliac disease is nowadays considered to be a systemic disorder. More and more diseases are proved to be associated with coeliac disease, in these conditions screening is strongly recommended. The studying of the recently explored autoantibodies against **tissue** transglutaminase brought us further in the understanding of the pathophysiology of coeliac disease. The spreading of reliable serologic methods modified our knowledge on the clinical picture and prevalence of the disease. In case of long-term dietary abuse the most common complications are decreased **bone** **mineral** density and development of lymphomas. Sustained glutenfree diet results in clinical and histological restitution, affects the course of associating diseases beneficially and decreases the risk for malignancies.

3/3,AB/26 (Item 26 from file: 155)
DIALOG(R)File 155:MEDLINE(R)

10907024 20471639 PMID: 11021631
Osteopontin.
Sodek J; Ganss B; McKee M D
MRC Group in Periodontal Physiology, Faculty of Dentistry, University of Toronto, ON, Canada. jaro.sodek@utoronto.ca
Critical reviews in oral biology and medicine : an official publication of the American Association of Oral Biologists (UNITED STATES) **2000**, 11 (3) p279-303, ISSN 1045-4411 Journal Code: 9009999
Document type: Journal Article; Review; Review, Tutorial
Languages: ENGLISH
Main Citation Owner: NLM
Record type: Completed
Osteopontin (OPN) is a highly phosphorylated sialoprotein that is a prominent component of the **mineralized** extracellular matrices of bones and teeth. OPN is characterized by the presence of a polyaspartic acid sequence and sites of Ser/Thr phosphorylation that mediate hydroxyapatite binding, and a highly conserved RGD motif that mediates cell attachment/signaling. Expression of OPN in a variety of tissues indicates a multiplicity of functions that involve one or more of these conserved motifs. While the lack of a clear phenotype in OPN "knockout" mice has not established a definitive role for OPN in any **tissue**, recent studies have provided some novel and intriguing insights into the versatility of this enigmatic protein in diverse biological events, including developmental processes, wound healing, immunological responses,

tumorigenesis, **bone** resorption, and calcification. The ability of OPN to stimulate cell activity through multiple receptors linked to several interactive signaling pathways can account for much of the functional diversity. In this **review**, we discuss the structural features of OPN that relate to its function in the formation, remodeling, and maintenance of bones and teeth.

3/3,AB/27 Item 27 from file: 155
DIALOG(R)File 155:MEDLINE(R)

10889072 20437033 PMID: 10983739
Raloxifene: a **review** of its use in postmenopausal osteoporosis.
Clemett D; Spencer C M
Adis International Limited, Mairangi Bay, Auckland, New Zealand.
Drugs (NEW ZEALAND) Aug **2000**, 60 (2) p379-411, ISSN 0012-6667
Journal Code: 7600076
Document type: Journal Article; Review; Review, Tutorial
Languages: ENGLISH
Main Citation Owner: NLM
Record type: Completed
Raloxifene is a selective estrogen receptor modulator that partially mimics the effects of estrogens in **bone** and the cardiovascular system, while functioning as an antiestrogen in endometrial and breast **tissue** . In randomised placebo-controlled studies involving postmenopausal women or patients with osteoporosis, raloxifene 60 to 150 mg/day was effective in increasing **bone mineral** density (BMD) over 12- to 36-month periods. At the 60 mg/day recommended dosage, increases of 1.6 to 3.4%, 0.9 to 2.3% and 1.0 to 1.6% were reported in lumbar spine, femoral neck and total hip, respectively, versus < or =0.5% with placebo. Raloxifene 60 or 120 mg/day decreased the risk of vertebral fractures over a 36-month period in postmenopausal patients with osteoporosis. Significant reductions in radiographic fracture risk versus placebo (30 and 50%) occurred regardless of whether patients had existing fractures at baseline. Although raloxifene did not affect the overall incidence of nonvertebral fractures, a reduction in the incidence of ankle fracture was reported in comparison with placebo. In postmenopausal women, raloxifene 60 mg/day significantly reduced serum levels of total and low density lipoprotein cholesterol from baseline, compared with placebo. High density lipoprotein cholesterol and triglyceride levels were unaffected. Raloxifene 60 or 120 mg/day reduced the risk of invasive breast cancer by 76% during a median of 40 months' follow-up in postmenopausal patients with osteoporosis and no history of breast cancer. A relative risk reduction of 90% was reported for estrogen-receptor positive invasive breast cancers; estrogen-receptor negative cancer risk was unaffected by raloxifene. Raloxifene was generally well tolerated in clinical trials at dosages up to 150 mg/day. Adverse events thought to be related to raloxifene treatment were hot flushes and leg cramps. Venous thromboembolism was the only serious adverse event thought to be related to raloxifene treatment and a relative risk of 3.1 compared with placebo treatment was reported in patients with osteoporosis. Vaginal bleeding occurred in < or =6.4% of raloxifene-treated women but was reported by 50 to 89% of those receiving estrogens or hormone replacement therapy (HRT). Raloxifene treatment was not associated with stimulatory effects on the endometrium. CONCLUSIONS: Raloxifene significantly increases BMD in postmenopausal women and reduces vertebral fracture risk in patients with osteoporosis. In clinical trials, raloxifene was generally well tolerated compared with placebo and HRT, although its propensity to cause hot flushes precludes use in women with vasomotor symptoms. In particular, the lack of stimulatory effects on the endometrium and the reduction in invasive breast cancer incidence indicate raloxifene as an attractive alternative to HRT for the management of postmenopausal osteoporosis.

3/3,AB/28 (Item 28 from file: 155)
DIALOG(R)File 155:MEDLINE(R)

10881339 20412854 PMID: 10958610
Application of vibrational spectroscopy to the study of **mineralized** tissues (**review**).
Carden A; Morris M D
Department of Chemistry, University of Michigan, Ann Arbor 48109-1055, USA.
Journal of biomedical optics (UNITED STATES) Jul **2000**, 5 (3) p259-68, ISSN 1083-3668 Journal Code: 9605853
Contract/Grant No.: T32 GM08353; GM; NIGMS
Document type: Journal Article; Review; Review, Tutorial
Languages: ENGLISH
Main Citation Owner: NLM
Record type: Completed
The infrared and Raman spectroscopy of **bone** and teeth tissues are reviewed. Characteristic spectra are obtained for both the **mineral** and protein components of these tissues. Vibrational spectroscopy is used to study the **mineralization** process, to define the chemical structure changes accompanying **bone** diseases, and to characterize interactions between prosthetic implants and tissues. Microspectroscopy allows acquisition of spatially resolved spectra, with micron scale resolution. Recently developed imaging modalities allow **tissue** imaging with chemical composition contrast.

3/3,AB/29 (Item 29 from file: 155)
DIALOG(R)File 155:MEDLINE(R)

10692013 20232233 PMID: 10769400
Role of pericytes in vascular calcification: a **review**.
Canfield A E; Doherty M J; Wood A C; Farrington C; Ashton B; Begum N; Harvey B; Poole A; Grant M E; Boot-Handford R P
Wellcome Trust Centre for Cell-Matrix Research, Schools of Medicine, Manchester, UK. ann.canfield@man.ac.uk
Zeitschrift fur Kardiologie (GERMANY) **2000**, 89 Suppl 2 p20-7, ISSN 0300-5860 Journal Code: 0360430
Document type: Journal Article; Review; Review, Tutorial
Languages: ENGLISH
Main Citation Owner: NLM
Record type: Completed
Pericytes are defined by their location in vivo; the pericyte partially surrounds the endothelial cell of the microvessel and shares a common basement membrane with it. As an integral part of the microvasculature, pericytes play a fundamental role in maintaining local and **tissue** homeostasis. Current evidence also suggests that pericytes function as progenitor cells capable of differentiating into a variety of different cell types including osteoblasts, chondrocytes and adipocytes. It is now apparent that cells resembling microvascular pericytes, and termed 'pericyte-like' cells, have a widespread distribution in vivo. Pericyte-like cells have been identified in the inner intima, the outer media, and in the vasa vasora of the adventitia of large, medium and small human arteries (1, 2). Moreover, recent studies have suggested that these cells may be responsible, at least in part, for mediating the calcification commonly associated with atherosclerosis (1, 3, 4). In this **review**, we a) examine the evidence that microvascular pericytes deposit a **bone**-like **mineralised** matrix in vitro, b) compare the morphological and biochemical properties of microvascular pericytes, calcifying vascular cells (CVCs) and 'classical' smooth muscle cells (SMCs) isolated from bovine aorta, c) demonstrate that microvascular pericytes

deposit a well organised matrix of **bone**, cartilage and fibrous **tissue** in vivo, and to discuss recent studies designed to gain a better understanding of how pericyte differentiation is regulated.

3/3,AB/30 Item 30 from file: 155
DIALOG(R)File 155:MEDLINE(R)

10612928 20125894 PMID: 10657279
Steroid hormone receptor expression and action in **bone**.
Bland R
Division of Medical Sciences, University of Birmingham, Edgbaston, Birmingham B15 2TT, U.K. R.Bland@bham.ac.uk
Clinical science (London, England : 1979) (ENGLAND) Feb **2000**, 98 (2) p217-40, ISSN 0143-5221 Journal Code: 7905731
Document type: Journal Article; Review; Review, Tutorial
Languages: ENGLISH
Main Citation Owner: NLM
Record type: Completed
The skeleton is a complex **tissue**, and hormonal control of **bone** remodelling is elaborate. The important role that steroid hormones play in **bone** cell development and in the maintenance of normal **bone** architecture is well established, but it is only relatively recently that it has become possible to describe their precise mechanism of action. This **review** focuses not only on the steroid hormones (oestrogens, corticosteroids, androgens and progesterone), but also on related hormones (vitamin D, thyroid hormone and the retinoids), all of which act via structurally homologous nuclear receptors that form part of the steroid/thyroid receptor superfamily. By examining the actions of all of these hormones in vivo and in vitro, this **review** gives a general overview of the current understanding of steroid hormone action in **bone**. In addition, a comprehensive **review** of steroid hormone receptor expression in **bone** cells is included. Finally, the role that future developments, such as steroid hormone receptor knockout mice, will play in our understanding of steroid hormone action in **bone** is considered.

3/3,AB/31 (Item 1 from file: 5)
DIALOG(R)File 5:Biosis Previews(R)

13935558 BIOSIS NO.: 200200564379
Molecular genetics of too much **bone**.
AUTHOR: Janssens Katrien; Van Hul Wim(a)
AUTHOR ADDRESS: (a)Department of Medical Genetics, University of Antwerp, Universiteitsplein 1, 2610, Antwerp**Belgium E-Mail: vnul@uia.ua.ac.be
JOURNAL: Human Molecular Genetics 11 (20):p2385-2393 **2002**
MEDIUM: print
ISSN: 0964-6906
DOCUMENT TYPE: Literature Review
RECORD TYPE: Abstract
LANGUAGE: English

ABSTRACT: **Bone** remodelling is an important process both throughout growth and in adult life. The homeostasis of **bone tissue** is maintained by the balanced processes of **bone** resorption and formation. Imbalance can give rise to a broad spectrum of skeletal pathologies, of which osteoporosis, characterized by a decrease in **bone** density leading to increased fracture risk, is the best known because of its high prevalence and consequently high socio-economic impact. At the opposite end of the spectrum, several genetic conditions displaying too much **bone** are situated. Mainly because of their

monogenic nature in contrast to the multifactorial character of osteoporosis the underlying molecular genetic causes for several of these conditions have been revealed recently. In this **review**, the most important gene identifications of the last years and their impact on the understanding of **bone** biology are discussed.

2002

3/3,AB/32 (Item 2 from file: 5)
DIALOG(R)File 5:Biosis Previews(R)

13935557 BIOSIS NO.: 200200564378
Molecular genetics of calcium sensing in **bone** cells.
AUTHOR: Purroy Jesus; Spurr Nigel K(a)
AUTHOR ADDRESS: (a)Discovery Genetics, GlaxoSmithKline, Five Moore Drive, PO Box 13398, Research Triangle Park, NC, 27709**USA E-Mail: nigelk spurr@gsk.com
JOURNAL: Human Molecular Genetics 11 (20):p2377-2384 **2002**
MEDIUM: print
ISSN: 0964-6906
DOCUMENT TYPE: Literature Review
RECORD TYPE: Abstract
LANGUAGE: English

ABSTRACT: The molecular mechanisms regulating **bone** remodelling are only partially understood. One of the controversial issues discussed during the past few years is the role that calcium signalling plays in this process and, in particular, in the functioning of the osteoclast. Calcium is involved in the recruitment and activation of osteoclasts and their subsequent detachment from **bone**. Parathyroid hormone and vitamin D are part of a systemic mechanism regulating calcium availability, storage and disposal. But there are conflicting results suggesting the presence of a local calcium-sensing mechanism in osteoclasts, in osteoblasts or in both. If this system could be characterized, it would be of therapeutic relevance for diseases such as postmenopausal osteoporosis and rheumatoid arthritis. Genetic data, animal models and cell-based assays have not yet been used to their full extent in this area. Here we **review** the available data and outline possible future strategies.

2002

3/3,AB/33 (Item 3 from file: 5)
DIALOG(R)File 5:Biosis Previews(R)

12986590 BIOSIS NO.: 200100193739
Dietary calcium and **bone** health in the elderly: Uncertainties about recommendations.
AUTHOR: Anderson John J B(a); Sjoberg H E
AUTHOR ADDRESS: (a)Department of Nutrition, Schools of Public Health and Medicine, University of North Carolina, Chapel Hill, NC, 27599-7400: jjb Anderson@unc.edu**USA
JOURNAL: Nutrition Research 21 (1-2):p263-268 January-February, **2001**
MEDIUM: print
ISSN: 0271-5317
DOCUMENT TYPE: Article
RECORD TYPE: Abstract
LANGUAGE: English
SUMMARY LANGUAGE: English

ABSTRACT: An adequate intake of calcium, and of other essential nutrients, has long been established to be necessary for the development and maintenance of **bone tissue**. A major question remains: how much calcium is needed across the life cycle in relation to other nutrients, to lifestyle factors, and to hereditary determinants. The 1997 publication on the calcium, the Dietary Recommended Intakes (DRIs) by the Institute of Medicine (IOM), includes recommendations for calcium intakes for males and females across the life cycle. The new guidelines for calcium consumption by the elderly are considerably higher than given in the tenth edition (1989) of the Recommended Dietary Guidelines (RDAs), i.e., 1200 mg per day compared to 800 mg per day. The rationale for the recommendations of increased dietary calcium are based on published reports in the literature. Current understandings of **bone** remodeling suggest that usual high calcium intakes could slow the rate of **bone** resorption through suppression of parathyroid hormone (PTH). Such a reduction in **bone** turnover could result in a modest but transient increase in BMD for a year or more. Presumably this high calcium intake would also diminish the formation of new **bone tissue**, as **bone** turnover is slowed. The major question regarding reduced turnover rates in elderly women (and men) following calcium supplementation is whether recently formed microfractures will be adequately replaced when PTH concentrations are so significantly reduced by the high calcium consumption. A brief **review** of the use of the multi-factorial approach to establishing rational nutrient intake guidelines for calcium, including the important role of physical activity in maintaining **bone** in the elderly, is offered. The Asian paradox of low-calcium intakes and low hip fracture rates is also considered. A goal of this **review** is to try to find a reasonable compromise on setting the calcium recommendation for elderly subjects in the US and Canada, and, perhaps, in much of the world. Whether meeting that goal requires the use of a daily supplement of calcium to achieve nutrient adequacy has not been established yet, but recent dietary intake surveys suggest that elderly American subjects, especially women, can not ingest even 800 mg of calcium per day without a supplement.

2001

3/3,AB/34 (Item 4 from file: 5)
DIALOG(R)File 5:Biosis Previews(R)

12892520 BIOSIS NO.: 200100099669
Factors associated with low **bone mineral** density in female patients with systemic lupus erythematosus.
AUTHOR: Lakshminarayanan Santhanam; Walsh Stephen; Mohanraj Mirna; Rothfield Naomi(a)
AUTHOR ADDRESS: (a)Department of Medicine, University of Connecticut Health Center, 263 Farmington Avenue, Farmington, CT, 06030-1310: Rothfield@NSO.UCHC.EDU**USA
JOURNAL: Journal of Rheumatology 28 (1):p102-108 January, **2001**
MEDIUM: print
ISSN: 0315-162X
DOCUMENT TYPE: Article
RECORD TYPE: Abstract
LANGUAGE: English
SUMMARY LANGUAGE: English

ABSTRACT: Objective. To study risk factors for low **bone mineral** density (BMD, g/cm) in patients with systemic lupus erythematosus (SLE). Methods: Ninety-two consecutive patients with SLE followed by rheumatology faculty between 1997 and 1999 completed a questionnaire regarding lifestyle during the clinic visit, a chart **review** was performed, and data were collected for the time of the first dual energy

x-ray absorptiometry (DXA) examination. Univariate and multivariate statistical analyses were used to assess relationships between various risk factors and BMD. Results: Ninety-eight percent of patients had received prednisone, 51% were postmenopausal (9 of whom received hormone replacement therapy), 68% had received hydroxychloroquine, and 15% were osteoporotic. The following factors were found to be significantly related to lower BMD by univariate analysis: Caucasian race, older age at diagnosis, higher age at the time of the first DXA, longer disease duration, higher cumulative corticosteroid dose, higher SLE Damage Index score, and postmenopausal status. In the multivariate analysis only the following factors were significant: Caucasian race, increased number of pregnancies, postmenopausal status, higher SLE Damage Index, and higher cumulative corticosteroid dose. An unexpected finding was that taking hydroxychloroquine was the only factor associated with higher BMD of the hip and spine in the univariate analysis, and it remained predictive of higher BMD of the hip and spine in the multivariate analysis. Conclusion: Hydroxychloroquine appears to protect against low BMD in corticosteroid treated patients with SLE.

2001

3/3,AB/35 (Item 5 from file: 5)
DIALOG(R)File 5:Biosis Previews(R)

12672591 BIOSIS NO.: 200000426093
Is enough attention being given to the adverse effects of corticosteroid therapy?.
AUTHOR: Hougardy D M C; Peterson G M(a); Bleasel M D; Randall C T C
AUTHOR ADDRESS: (a)Tasmanian School of Pharmacy, Faculty of Health Science, University of Tasmania, Hobart, Tasmania, 7001**Australia
JOURNAL: Journal of Clinical Pharmacy and Therapeutics 25 (3):p227-234 June, **2000**
MEDIUM: print
ISSN: 0269-4727
DOCUMENT TYPE: Literature Review
RECORD TYPE: Abstract
LANGUAGE: English
SUMMARY LANGUAGE: English

ABSTRACT: Background: Although the corticosteroids are valuable anti-inflammatory and immunosuppressive agents, they also possess many potential adverse effects, especially with continued use. In particular, long-term corticosteroid exposure carries a significant risk of osteoporosis. Aim: To **review** the use of corticosteroids in patients presenting to the major teaching hospital in Tasmania, Australia; principally to determine whether patients receiving long-term corticosteroid therapy were being monitored for loss of **bone mineral** density and offered preventive therapy for osteoporosis. Methods: A retrospective **review** of the medical records for 212 consecutive patients admitted to the medical wards of the hospital over a 6-month period and receiving treatment with either oral or inhaled corticosteroids, was performed. An extensive range of demographic and clinical variables was recorded for each patient. Patients were also questioned about diet and exercise, and whether they had undergone tests for measuring **bone mineral** density or blood glucose. Results: The median age of the patients was 69 years (range: 15-90 years) and 58% were female. Over half (53%) of the patients were on oral corticosteroids only, with 26% using inhaled corticosteroids only, and 21% on both oral and inhaled corticosteroid therapy. The most common conditions for which patients were receiving corticosteroid therapy were asthma (37% of patients), chronic obstructive pulmonary disease (33%) and rheumatoid arthritis (17%). The most commonly used oral corticosteroid was

prednisolone (93%), the median daily dose was 10 mg prednisolone equivalent, and the median duration of oral corticosteroid treatment was 50 weeks. Disregarding short courses, the median duration of oral corticosteroid treatment was 104 weeks. Almost one-third (31%) of the patients receiving oral corticosteroid treatment had been taking the equivalent of 7.5 mg prednisolone daily for at least 6 months. Only 11% of all patients on oral corticosteroids and 21% of those who had been taking oral corticosteroids for at least one year had documented evidence of **bone mineral** density testing being performed in the past in the hospital. Only 21% of all patients on oral corticosteroids and 31% of those who had been taking oral corticosteroids for at least one year were receiving medication for osteoporosis prevention, and only 15% of women over 45 years of age and on oral corticosteroid therapy were taking hormone replacement therapy. Only about half of the patients on long-term systemic corticosteroid therapy had either documented evidence in their hospital medical records, or were aware, of having undergone blood glucose testing in the preceding 12 months. Conclusions: More attention to the prevention and monitoring of possible adverse effects of long-term corticosteroid therapy is warranted. Guidelines covering preventive measures and treatment options for corticosteroid induced osteoporosis need to be considered routinely when using these agents.

2000

3/3,AB/36 (Item 1 from file: 444)
DIALOG(R)File 444:New England Journal of Med.

00123423

Current Concepts: Corticosteroid Insufficiency in Acutely Ill Patients (**Review** Article)

Cooper, Mark S.; Stewart, Paul M.
The New England Journal of Medicine
Feb 20, **2003**; 348 (8),pp 727-734
LINE COUNT: 00391 WORD COUNT: 05406

3/3,AB/37 (Item 2 from file: 444)
DIALOG(R)File 444:New England Journal of Med.

00123396

Drug Therapy: Selective Estrogen-Receptor Modulators -- Mechanisms of Action and Application to Clinical Practice (**Review** Article)

Riggs, B. Lawrence; Hartmann, Lynn C.
The New England Journal of Medicine
Feb 13, **2003**; 348 (7),pp 618-629
LINE COUNT: 00537 WORD COUNT: 07420

3/3,AB/38 (Item 3 from file: 444)
DIALOG(R)File 444:New England Journal of Med.

00123317

Serum Retinol Levels and the Risk of Fracture Original Articles

Michaelsson, Karl; Lithell, Hans; Vessby, Bengt; Melhus, Hakan.
The New England Journal of Medicine
Jan 23, **2003**; 348 (4),pp 287-294
LINE COUNT: 00374   WORD COUNT: 05169

Abstract
Background: Although studies in animals and epidemiologic studies have indicated that a high vitamin A intake is associated with increased **bone** fragility, no biologic marker of vitamin A status has thus far been used to assess the risk of fractures in humans.

Methods: We enrolled 2322 men, 49 to 51 years of age, in a population-based, longitudinal cohort study. Serum retinol and beta carotene were analyzed in samples obtained at enrollment. Fractures were documented in 266 men during 30 years of follow-up. Cox regression analysis was used to determine the risk of fracture according to the serum retinol level.

Results: The risk of fracture was highest among men with the highest levels of serum retinol. Multivariate analysis of the risk of fracture in the highest quintile for serum retinol (>75.62 microg per deciliter 2.64 micromol per liter]) as compared with the middle quintile (62.16 to 67.60 microg per deciliter 2.17 to 2.36 micromol per liter]) showed that the rate ratio was 1.64 (95 percent confidence interval, 1.12 to 2.41) for any fracture and 2.47 (95 percent confidence interval, 1.15 to 5.28) for hip fracture. The risk of fracture was further increased within the highest quintile for serum retinol. Men with retinol levels in the 99th percentile (>103.12 microg per deciliter 3.60 micromol per liter]) had an overall risk of fracture that exceeded the risk among men with lower levels by a factor of seven (P<0.001). The level of serum beta carotene was not associated with the risk of fracture.

Conclusions: Our findings, which are consistent with the results of studies in animals, as well as in vitro and epidemiologic dietary studies, suggest that current levels of vitamin A supplementation and food fortification in many Western countries may need to be reassessed.

N Engl J Med 2003;348:287-94.

3/3,AB/39   (Item 4 from file: 444)
DIALOG(R)File 444:New England Journal of Med.

00123279

Weekly Clinicopathological Exercises: Case 1-2003: A 43-Year-Old Man with Fever and Night Sweats (Case Records of the Massachusetts General Hospital)

Tanoue, Lynn T.; Mark, Eugene J.
The New England Journal of Medicine
Jan 9, **2003**; 348 (2),pp 151-161
LINE COUNT: 00609   WORD COUNT: 08415

3/3,AB/40   (Item 5 from file: 444)
DIALOG(R)File 444:New England Journal of Med.

00123251

Preventing Falls in Elderly Persons Clinical Practice

Tinetti, Mary E.

The New England Journal of Medicine
Jan 2, **2003**; 348 (1),pp 42-49
LINE COUNT: 00285 WORD COUNT: 03933

3/3,AB/41 (Item 6 from file: 444)
DIALOG(R)File 444:New England Journal of Med.

00122729

Celiac Sprue (Correspondence)

Colli, Agostino; Colucci, Alice; Conte, Dario; Gomollon, Fernando; Tribole, Evelyn; Kupper, Cynthia; Pietzak, Michelle; Spanier, Bernhard W.M.; Dietz, Barbara; Mulder, Chris J.J.; Farrell, Richard J.; Kelly, Ciaran P.
The New England Journal of Medicine
Aug 8, **2002**; 347 (6),pp 446-448
LINE COUNT: 00132 WORD COUNT: 01831

3/3,AB/42 (Item 7 from file: 444)
DIALOG(R)File 444:New England Journal of Med.

00122717

Medical Progress: Polymyalgia Rheumatica and Giant-Cell Arteritis (**Review** Article)

Salvarani, Carlo; Cantini, Fabrizio; Boiardi, Luigi; Hunder, Gene G.
The New England Journal of Medicine
Jul 25, **2002**; 347 (4),pp 261-271
LINE COUNT: 00499 WORD COUNT: 06888

3/3,AB/43 (Item 8 from file: 444)
DIALOG(R)File 444:New England Journal of Med.

00122681

Osteoprotegerin Deficiency and Juvenile Paget's Disease (Original Articles)

Whyte, Michael P.; Obrecht, Sara E.; Finnegan, Patrick M.; Jones, Jonathan L.; Podgornik, Michelle N.; McAlister, William H.; Mumm, Steven.
The New England Journal of Medicine
Jul 18, **2002**; 347 (3),pp 175-184
LINE COUNT: 00462 WORD COUNT: 06387

Abstract
Background: Juvenile Paget's disease, an autosomal recessive osteopathy, is characterized by rapidly remodeling woven **bone**, osteopenia, fractures, and progressive skeletal deformity. The molecular basis is not known. Osteoprotegerin deficiency could explain juvenile Paget's disease because osteoprotegerin suppresses **bone** turnover by functioning as a decoy receptor for osteoclast differentiation factor (also called RANK ligand).
Methods: We evaluated two apparently unrelated Navajo patients with

juvenile Paget's disease for defects in the gene encoding osteoprotegerin (TNFRSF11B) using polymerase-chain reaction (PCR) amplification followed by direct sequencing and Southern blotting of genomic DNA. Genetic markers near TNFRSF11B were evaluated by both a PCR method that involved sequence tagged site-content mapping of a deletion of TNFRSF11B and PCR spanning the DNA break points.

Results: Both patients had a homozygous deletion of TNFRSF11B, with identical break points, on chromosome 8q24.2. The defect spans approximately 100 kb, but neighboring genes are intact. We found that serum levels of osteoprotegerin and soluble osteoclast differentiation factor were undetectable and markedly increased, respectively.

Conclusions: Juvenile Paget's disease can result from osteoprotegerin deficiency caused by homozygous deletion of TNFRSF11B. (N Engl J Med 2002;347:175-84.)

3/3,AB/44 (Item 9 from file: 444)
DIALOG(R)File 444:New England Journal of Med.

00122437

One Foot Away (Clinical Problem-Solving)

Haberer, Jessica E.; Ix, Joachim H.; Tierney, Lawrence M., Jr.
The New England Journal of Medicine
May 2, **2002**; 346 (18),pp 1394-1397
LINE COUNT: 00336 WORD COUNT: 04644

3/3,AB/45 (Item 10 from file: 444)
DIALOG(R)File 444:New England Journal of Med.

00122332

Estrogen-Receptor Polymorphisms and Effects of Estrogen Replacement on High-Density Lipoprotein Cholesterol in Women with Coronary Disease (Original Articles)

Herrington, David M.; Howard, Timothy D.; Hawkins, Gregory A.; Reboussin, David M.; Xu, Jianfeng; Zheng, Siqun L.; Brosnihan, K. Bridget; Meyers, Deborah A.; Bleecker, Eugene R.
The New England Journal of Medicine
Mar 28, **2002**; 346 (13),pp 967-974
LINE COUNT: 00362 WORD COUNT: 05002

Abstract

Background: Sequence variants in the gene encoding estrogen receptor (alpha) (ER-(alpha)) may modify the effects of hormone-replacement therapy on levels of high-density lipoprotein (HDL) cholesterol and other outcomes related to estrogen treatment in postmenopausal women.

Methods: We characterized 309 women with coronary artery disease who were enrolled in the Estrogen Replacement and Atherosclerosis trial with respect to eight previously described and two newly identified ER-(alpha) polymorphisms, and we examined the association between these polymorphisms and the response of HDL cholesterol and other lipids to treatment with estrogen alone or estrogen plus progestin.

Results: After adjustment for age, race, diabetes status, body-mass index, smoking status, alcohol intake, and frequency of exercise, the 18.9 percent of the women who had the IVS1-401 C/C genotype (i.e., with C on both chromosomes in intervening sequence 1 at position 401 before exon 2

had an increase in the HDL cholesterol level with hormone-replacement therapy that was more than twice the increase observed in the other women (13.1 mg per deciliter vs. 6.0 mg per deciliter, P for treatment-by-genotype interaction = 0.004); this effect was limited to changes in the HDL subfraction 3 (HDL sub 3) (P for interaction = 0.04). Similar patterns of response were observed for three other highly linked ER- alpha intron 1 polymorphisms close to the IVS1-401 site (range of P values for interaction = 0.07 to 0.005). The pattern of increased response of HDL cholesterol in women with the IVS1-401 C/C genotype was evident in both the women receiving estrogen and those receiving estrogen plus progestin, was preserved across racial and ethnic groups, and was significant among women who were compliant with the study medication (P<0.001).

Conclusions: Postmenopausal women with coronary disease who have the ER- alpha) IVS1-401 C/C genotype, or several other closely related genotypes, have an augmented response of HDL cholesterol to hormone-replacement therapy. (N Engl J Med 2002;346:967-74.)

3/3,AB/46 (Item 11 from file: 444)

00122247

Intravenous Zoledronic Acid in Postmenopausal Women with Low **Bone Mineral** Density (Original Articles)

Reid, Ian R.; Brown, Jacques P.; Burckhardt, Peter; Horowitz, Zebulun; Richardson, Peter; Trechsel, Ulrich; Widmer, Albert; Devogelaer, Jean-Pierre; Kaufman, Jean-Marc; Jaeger, Philippe; Body, Jean-Jacques; Meunier, Pierre J.
The New England Journal of Medicine
Feb 28, **2002**; 346 (9), pp 653-661
LINE COUNT: 00362 WORD COUNT: 05006

Abstract

Background: Bisphosphonates are effective agents for the management of osteoporosis. Their low bioavailability and low potency necessitate frequent administration on an empty stomach, which may reduce compliance. Gastrointestinal intolerance limits maximal dosing. Although intermittent intravenous treatments have been used, the optimal doses and dosing interval have not been systematically explored.

Methods: We studied the effects of five regimens of zoledronic acid, the most potent bisphosphonate, on **bone** turnover and density in 351 postmenopausal women with low **bone mineral** density in a one-year, randomized, double-blind, placebo-controlled trial. Women received placebo or intravenous zoledronic acid in doses of 0.25 mg, 0.5 mg, or 1 mg at three-month intervals. In addition, one group received a total annual dose of 4 mg as a single dose, and another received two doses of 2 mg each, six months apart. Lumbar-spine **bone mineral** density was the primary end point.

Results: There were similar increases in **bone mineral** density in all the zoledronic acid groups to values for the spine that were 4.3 to 5.1 percent higher than those in the placebo group (P<0.001) and values for the femoral neck that were 3.1 to 3.5 percent higher than those in the placebo group (P<0.001). Biochemical markers of **bone** resorption were significantly suppressed throughout the study in all zoledronic acid groups. Myalgia and pyrexia occurred more commonly in the zoledronic acid groups, but treatment-related dropout rates were similar to that in the placebo group.

Conclusions: Zoledronic acid infusions given at intervals of up to one year produce effects on **bone** turnover and **bone** density as great

as those achieved with daily oral dosing with bisphosphonates with proven efficacy against fractures, suggesting that an annual infusion of zoledronic acid might be an effective treatment for postmenopausal osteoporosis. N Engl J Med 2002;346:653-61.

3/3,AB/47 (Item 12 from file: 444)
DIALOG(R)File 444:New England Journal of Med.

00122081

Paternally Inherited Inactivating Mutations of the GNAS1 Gene in Progressive Osseous Heteroplasia (Original Articles)

Shore, Eileen M.; Ahn, Jaimo; de Beur, Suzanne Jan; Li, Ming; Xu, Meiqi; Gardner, R.J. McKinlay; Zasloff, Michael A.; Whyte, Michael P.; Levine, Michael A.; Kaplan, Frederick S.
The New England Journal of Medicine
Jan 10, **2002**; 346 (2),pp 99-106
LINE COUNT: 00358 WORD COUNT: 04948

Abstract
Background: Progressive osseous heteroplasia (POH), an autosomal dominant disorder, is characterized by extensive dermal ossification during childhood, followed by disabling and widespread heterotopic ossification of skeletal muscle and deep connective **tissue**. Occasional reports of mild heterotopic ossification in Albright's hereditary osteodystrophy (AHO) and a recent report of two patients with AHO who had atypically extensive heterotopic ossification suggested a common genetic basis for the two disorders. AHO is caused by heterozygous inactivating mutations in the GNAS1 gene that result in decreased expression or function of the alpha subunit of the stimulatory G protein (G(sub s)(alpha)) of adenylyl cyclase.
Methods: We tested the hypothesis that GNAS1 mutations cause POH, using the polymerase chain reaction to amplify GNAS1 exons and exon-intron boundaries in 18 patients with sporadic or familial POH.
Results: Heterozygous inactivating GNAS1 mutations were identified in 13 of the 18 probands with POH. The defective allele in POH is inherited exclusively from fathers, a result consistent with a model of imprinting for GNAS1. Direct evidence that the same mutation can cause either POH or AHO was observed within a single family, in which the phenotype correlated with the parental origin of the mutant allele.
Conclusions: Paternally inherited inactivating GNAS1 mutations cause POH. This finding extends the range of phenotypes derived from haploinsufficiency of GNAS1, provides evidence that imprinting is a regulatory mechanism for GNAS1 expression, and suggests that G(sub s)(alpha) is a critical negative regulator of osteogenic commitment in nonosseous connective tissues. (N Engl J Med 2002;346:99-106.)

3/3,AB/48 (Item 13 from file: 444)
DIALOG(R)File 444:New England Journal of Med.

00122027

What Vitamins Should I Be Taking, Doctor? (Clinical Practice)

Willett, Walter C.; Stampfer, Meir J.
The New England Journal of Medicine
Dec 20, **2001**; 345 (25),pp 1819-1824
LINE COUNT: 00343 WORD COUNT: 04742

3/3,AB/49 (Item 14 from file: 444)
DIALOG(R)File 444:New England Journal of Med.

00121830

Weekly Clinicopathological Exercises: Case 31-2001: A 70-Year-Old Woman with End-Stage Renal Disease and Cutaneous Ulcers Case Records of the Massachusetts General Hospital

Baran, Daniel T.; Letts, Gary St.A.
The New England Journal of Medicine
Oct 11, **2001**; 345 (15),pp 1119-1124
LINE COUNT: 00306 WORD COUNT: 04231

3/3,AB/50 (Item 15 from file: 444)
DIALOG(R)File 444:New England Journal of Med.

00121787

Pamidronate to Prevent **Bone** Loss during Androgen-Deprivation Therapy for Prostate Cancer (Original Articles)

Smith, Matthew R.; McGovern, Francis J.; Zietman, Anthony L.; Fallon, Mary Anne; Hayden, Douglas L.; Schoenfeld, David A.; Kantoff, Philip W.; Finkelstein, Joel S.
The New England Journal of Medicine
Sep 27, **2001**; 345 (13),pp 948-955
LINE COUNT: 00279 WORD COUNT: 03859

Abstract
Background: Treatment with a gonadotropin-releasing hormone agonist decreases **bone mineral** density and increases the risk of fracture in men with prostate cancer. We conducted a controlled study of the prevention of osteoporosis in men undergoing treatment with a gonadotropin-releasing hormone agonist.
Methods: In a 48-week, open-label study, we randomly assigned 47 men with advanced or recurrent prostate cancer and no **bone** metastases to receive either leuprolide alone or leuprolide and pamidronate (60 mg intravenously every 12 weeks). **Bone mineral** density of the lumbar spine and the proximal femur was measured by dual-energy x-ray absorptiometry. Trabecular **bone mineral** density of the lumbar spine was measured by quantitative computed tomography. Forty-one men completed the study.
Results: In men treated with leuprolide alone, the mean (+/-SE) **bone mineral** density decreased by 3.3+/-0.7 percent in the lumbar spine, 2.1+/-0.6 percent in the trochanter, and 1.8+/-0.4 percent in the total hip, and the mean trabecular **bone mineral** density of the lumbar spine decreased by 8.5+/-1.8 percent (P<0.001 for each comparison with the base-line value). In contrast, the mean **bone mineral** density did not change significantly at any skeletal site in men treated with both leuprolide and pamidronate. There were significant differences between the two groups in the mean changes in **bone mineral** density at 48 weeks in the lumbar spine (P<0.001), trochanter (P=0.003), total hip (P=0.005), and trabecular **bone** of the lumbar spine (P=0.02).
Conclusions: Pamidronate prevents **bone** loss in the hip and lumbar spine in men receiving treatment for prostate cancer with a

gonadotropin-releasing hormone agonist. N Engl J Med 2001;345:948-55.

3/3,AB/51 (Item 16 from file: 444)
DIALOG(R)File 444:New England Journal of Med.

00121773

Weekly Clinicopathological Exercises: Case 29-2001: A 14-Year-Old Boy with Abnormal Bones and a Sacral Mass (Case Records of the Massachusetts General Hospital)

Terek, Richard M.; Nielsen, G. Petur.
The New England Journal of Medicine
Sep 20, **2001**; 345 (12),pp 903-908
LINE COUNT: 00357 WORD COUNT: 04927

3/3,AB/52 (Item 17 from file: 444)
DIALOG(R)File 444:New England Journal of Med.

00121680

Subclinical Hyperthyroidism (Clinical Practice)

Toft, Anthony D.
The New England Journal of Medicine
Aug 16, **2001**; 345 (7),pp 512-516
LINE COUNT: 00307 WORD COUNT: 04249

3/3,AB/53 (Item 18 from file: 444)
DIALOG(R)File 444:New England Journal of Med.

00121613

Weekly Clinicopathological Exercises: Case 23-2001: An 18-Month-Old Girl with Persistent Diarrhea and Malnutrition (Case Records of the Massachusetts General Hospital)

Duggan, Christopher P.; Misdraji, Joseph.
The New England Journal of Medicine
Jul 26, **2001**; 345 (4),pp 276-281
LINE COUNT: 00382 WORD COUNT: 05275

3/3,AB/54 (Item 19 from file: 444)
DIALOG(R)File 444:New England Journal of Med.

00121612

Drug Therapy: Treatment of Endometriosis (**Review** Article)

Olive, David L.; Pritts, Elizabeth A.
The New England Journal of Medicine
Jul 26, **2001**; 345 (4),pp 266-275

LINE COUNT: 00478   WORD COUNT: 06597

3/3,AB/55   (Item 20 from file: 444)
DIALOG(R)File 444:New England Journal of Med.

00121519

Drug Therapy: Side Effects of Adjuvant Treatment of Breast Cancer
Review Articles)

Shapiro, Charles L.; Recht, Abram.
The New England Journal of Medicine
Jun 28, **2001**; 344 (26),pp 1997-2008
LINE COUNT: 00570   WORD COUNT: 07867

3/3,AB/56   (Item 21 from file: 444)
DIALOG(R)File 444:New England Journal of Med.

00121403

Weekly Clinicopathological Exercises: Case 15-2001: An 72-Year-Old Man with Persistent Fever and Hypotension (Case Records of the Massachusetts General Hospital)

Kaiser, Ursula B.; Hedley-Whyte, E. Tessa.
The New England Journal of Medicine
May 17, **2001**; 344 (20),pp 1536-1542
LINE COUNT: 00332   WORD COUNT: 04584

3/3,AB/57   (Item 22 from file: 444)
DIALOG(R)File 444:New England Journal of Med.

00121369

Effect of Parathyroid Hormone (1-34) on Fractures and **Bone Mineral** Density in Postmenopausal Women with Osteoporosis (Original Articles)

Neer, Robert M.; Arnaud, Claude D.; Zanchetta, Jose R.; Prince, Richard; Gaich, Gregory A.; Reginster, Jean-Yves; Hodsman, Anthony B.; Eriksen, Erik F.; Ish-Shalom, Sophia; Genant, Harry K.; Wang, Ouhong; Bruce H. Mitlak.
The New England Journal of Medicine
May 10, **2001**; 344 (19),pp 1434-1441
LINE COUNT: 00373   WORD COUNT: 05155

Abstract
Background: Once-daily injections of parathyroid hormone or its amino-terminal fragments increase **bone** formation and **bone** mass without causing hypercalcemia, but their effects on fractures are unknown.
Methods: We randomly assigned 1637 postmenopausal women with prior vertebral fractures to receive 20 or 40 microg of parathyroid hormone (1-34) or placebo, administered subcutaneously by the women daily. We obtained vertebral radiographs at base line and at the end of the study (median duration of observation, 21 months) and performed serial

measurements of **bone** mass by dual-energy x ray absorptiometry.

Results. New vertebral fractures occurred in 14 percent of the women in the placebo group and in 5 percent and 4 percent, respectively, of the women in the 20-microg and 40-microg parathyroid hormone groups; the respective relative risks of fracture in the 20-microg and 40-microg groups, as compared with the placebo group, were 0.35 and 0.31 (95 percent confidence intervals, 0.22 to 0.55 and 0.19 to 0.50). New nonvertebral fragility fractures occurred in 6 percent of the women in the placebo group and in 3 percent of those in each parathyroid hormone group (relative risk, 0.47 and 0.46, respectively [95 percent confidence intervals, 0.25 to 0.88 and 0.25 to 0.86]). As compared with placebo, the 20-microg and 40-microg doses of parathyroid hormone increased **bone mineral** density by 9 and 13 more percentage points in the lumbar spine and by 3 and 6 more percentage points in the femoral neck; the 40-microg dose decreased **bone mineral** density at the shaft of the radius by 2 more percentage points. Both doses increased total-body **bone mineral** by 2 to 4 more percentage points than did placebo. Parathyroid hormone had only minor side effects (occasional nausea and headache).

Conclusions: Treatment of postmenopausal osteoporosis with parathyroid hormone (1-34) decreases the risk of vertebral and nonvertebral fractures; increases vertebral, femoral, and total-body **bone mineral** density; and is well tolerated. The 40-microg dose increased **bone mineral** density more than the 20-microg dose but had similar effects on the risk of fracture and was more likely to have side effects. (N Engl J Med 2001;344:1434-41.)

3/3,AB/58 (Item 23 from file: 444)
DIALOG(R)File 444:New England Journal of Med.

00121067

Mechanisms of Disease: Estrogen and the Risk of Breast Cancer (**Review** Article)

Clemons, Mark; Goss, Paul.
The New England Journal of Medicine
Jan 25, **2001**; 344 (4),pp 276-285
LINE COUNT: 00428 WORD COUNT: 05914

3/3,AB/59 (Item 24 from file: 444)
DIALOG(R)File 444:New England Journal of Med.

00120971

Medical Progress: Hyperparathyroid and Hypoparathyroid Disorders (**Review** Articles)

Marx, Stephen J.
The New England Journal of Medicine
Dec 21, **2000**; 343 (25),pp 1863-1875
LINE COUNT: 00624 WORD COUNT: 08623

3/3,AB/60 (Item 25 from file: 444)
DIALOG(R)File 444:New England Journal of Med.

00120876

Weekly Clinicopathological Exercises Case 35-2000: An 82 Year-Old Woman with Bilateral Adrenal Masses and Low-Grade Fever Case Records of the Massachusetts General Hospital

Udelsman, Robert; Dong, Henry Y.
The New England Journal of Medicine
Nov 16, 2000; 343 (20),pp 1477-1483
LINE COUNT: 00403 WORD COUNT: 05570

3/3,AB/61 (Item 26 from file: 444)
DIALOG(R)File 444:New England Journal of Med.

00120853

Prevention of Hip Fracture in Elderly People with Use of a Hip Protector (Original Articles)

Kannus, Pekka; Parkkari, Jari; Niemi, Seppo; Pasanen, Matti; Palvanen, Mika; Jarvinen, Markku; Vuori, Ilkka.
The New England Journal of Medicine
Nov 23, 2000; 343 (21),pp 1506-1513
LINE COUNT: 00375 WORD COUNT: 05178

Abstract

Background: Hip fractures are common in frail elderly adults worldwide. We investigated the effect of an anatomically designed external hip protector on the risk of these age-related fractures.

Methods: We randomly assigned 1801 ambulatory but frail elderly adults (1409 women and 392 men; mean age, 82 years), in a 1:2 ratio, either to a group that wore a hip protector or to a control group. Fractures of the hip and all other fractures were recorded until the end of the first full month after 62 hip fractures had occurred in the control group. The risk of fracture in the two groups was compared, and in the hip-protector group the risk of fracture was also analyzed according to whether the protector had been in use at the time of a fall.

Results: During follow-up, 13 subjects in the hip-protector group had a hip fracture, as compared with 67 subjects in the control group. The respective rates of hip fracture were 21.3 and 46.0 per 1000 person-years (relative hazard in the hip-protector group, 0.4; 95 percent confidence interval, 0.2 to 0.8; P=0.008). The risk of pelvic fracture was slightly but not significantly lower in the hip-protector group than in the control group (2 subjects and 12 subjects, respectively, had pelvic fracture) (relative hazard, 0.4; 95 percent confidence interval, 0.1 to 1.8; P greater/equal 0.05). The risk of other fractures was similar in the two groups. In the hip-protector group, four subjects had a hip fracture (among 1034 falls) while wearing the protector, and nine subjects had a hip fracture (among 370 falls) while not wearing the protector (relative hazard, 0.2; 95 percent confidence interval, 0.05 to 0.5; P=0.002).

Conclusions: The risk of hip fracture can be reduced in frail elderly adults by the use of an anatomically designed external hip protector. (N Engl J Med 2000;343:1506-13.)

3/3,AB/62 (Item 27 from file: 444)
DIALOG(R)File 444:New England Journal of Med.

00120766

Weekly Clinicopathological Exercises: Case 32 2000: A Boy with Vertebral Compression Fractures Case Records of the Massachusetts General Hospital

Shapiro, Frederic D.; de Leval, Laurence.
The New England Journal of Medicine
Oct 19, **2000**; 343 16 ,pp 1168-1176
LINE COUNT: 00396 WORD COUNT: 05467

3/3,AB/63 (Item 28 from file: 444)
DIALOG R File 444:New England Journal of Med.

00120498

Primary Care: Reducing the Risk of Breast Cancer (**Review** Article)

Chlebowski, Rowan T.
The New England Journal of Medicine
Jul 20, **2000**; 343 (3),pp 191-198
LINE COUNT: 00393 WORD COUNT: 05428

3/3,AB/64 (Item 29 from file: 444)
DIALOG(R)File 444:New England Journal of Med.

00120401

A Comparison of Observational Studies and Randomized, Controlled Trials (Special Articles)

Benson, Kjell; Hartz, Arthur J.
The New England Journal of Medicine
Jun 22, **2000**; 342 (25),pp 1878-1886
LINE COUNT: 00347 WORD COUNT: 04801

Abstract

Background: For many years it has been claimed that observational studies find stronger treatment effects than randomized, controlled trials. We compared the results of observational studies with those of randomized, controlled trials.

Methods: We searched the Abridged Index Medicus and Cochrane data bases to identify observational studies reported between 1985 and 1998 that compared two or more treatments or interventions for the same condition. We then searched the Medline and Cochrane data bases to identify all the randomized, controlled trials and observational studies comparing the same treatments for these conditions. For each treatment, the magnitudes of the effects in the various observational studies were combined by the Mantel-Haenszel or weighted analysis-of-variance procedure and then compared with the combined magnitude of the effects in the randomized, controlled trials that evaluated the same treatment.

Results: There were 136 reports about 19 diverse treatments, such as calcium-channel-blocker therapy for coronary artery disease, appendectomy, and interventions for subfertility. In most cases, the estimates of the treatment effects from observational studies and randomized, controlled trials were similar. In only 2 of the 19 analyses of treatment effects did the combined magnitude of the effect in observational studies lie outside the 95 percent confidence interval for the combined magnitude in the randomized, controlled trials.

Conclusions: We found little evidence that estimates of treatment

effects in observational studies reported after 1984 are either consistently larger than or qualitatively different from those obtained in randomized, controlled trials. N Engl J Med 2000;342:1878-86.

3/3,AB/65 (Item 30 from file: 444)
DIALOG(R)File 444:New England Journal of Med.

00120186

Coronary-Artery Calcification in Young Adults with End-Stage Renal Disease Who Are Undergoing Dialysis (Original Articles)

Goodman, William G.; Goldin, Jonathan; Kuizon, Beatriz D.; Yoon, Chun; Gales, Barbara; Sider, Donna; Wang, Yan; Chung, Joanie; Emerick, Aletha; Greaser, Lloyd; Elashoff, Robert M.; Salusky, Isidro B.
The New England Journal of Medicine
May 18, **2000**; 342 (20),pp 1478-1483
LINE COUNT: 00384 WORD COUNT: 05312

Abstract
Background: Cardiovascular disease is common in older adults with end-stage renal disease who are undergoing regular dialysis, but little is known about the prevalence and extent of cardiovascular disease in children and young adults with end-stage renal disease.
Methods: We used electron-beam computed tomography (CT) to screen for coronary-artery calcification in 39 young patients with end-stage renal disease who were undergoing dialysis (mean +/-SD] age, 19+/-7 years; range, 7 to 30) and 60 normal subjects 20 to 30 years of age. In those with evidence of calcification on CT scanning, we determined its extent. The results were correlated with the patients' clinical characteristics, serum calcium and phosphorus concentrations, and other biochemical variables.
Results: None of the 23 patients who were younger than 20 years of age had evidence of coronary-artery calcification, but it was present in 14 of the 16 patients who were 20 to 30 years old. Among those with calcification, the mean calcification score was 1157+/-1996, and the median score was 297. By contrast, only 3 of the 60 normal subjects had calcification. As compared with the patients without coronary-artery calcification, those with calcification were older (26+/-3 vs. 15+/-5 years, P<0.001) and had been undergoing dialysis for a longer period (14+/-5 vs. 4+/-4 years, P<0.001). The mean serum phosphorus concentration, the mean calcium-phosphorus ion product in serum, and the daily intake of calcium were higher among the patients with coronary-artery calcification. Among 10 patients with calcification who underwent follow-up CT scanning, the calcification score nearly doubled (from 125+/-104 to 249+/-216, P=0.02) over a mean period of 20+/-3 months.
Conclusions: Coronary-artery calcification is common and progressive in young adults with end-stage renal disease who are undergoing dialysis. (N Engl J Med 2000;342:1478-83.)

3/3,AB/66 (Item 31 from file: 444)
DIALOG(R)File 444:New England Journal of Med.

00120024

Weekly Clinicopathological Exercises: Case 4-2000: A 64-Year-Old Man with Cushing's Syndrome and a Pancreatic Mass (Case Records of the Massachusetts General Hospital

Brandt, Lawrence J.; Mark, Eugene J.
The New England Journal of Medicine
Feb 10, 2000; 342 6 ,pp 414-420
LINE COUNT: 00470 WORD COUNT: 06494

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S1        1025   BONE AND TISSUE AND REVIEW AND PY>1999
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          165957  REPAIR
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 6/3,AB/1     (Item 1 from file: 155)
DIALOG(R)File 155:MEDLINE(R)
(c) format only 2003 The Dialog Corp. All rts. reserv.

13944761   22195138   PMID: 12205411
  Overview of bone morphogenetic proteins.
  Wozney John M; et al
  Wyeth Research, Cambridge, Massachusetts 02140, USA. jwozney@wyeth.com
  Spine (United States)   Aug 15 2002,  27  (16 Suppl 1)  pS2-8,
ISSN 1528-1159   Journal Code: 7610646
  Document type: Journal Article; Review; Review, Tutorial
  Languages: ENGLISH
  Main Citation Owner: NLM
  Record type: Completed
```

STUDY DESIGN: A literature **review** was conducted. OBJECTIVES: To **review** the discovery of the **bone** morphogenetic proteins and describe the **bone** morphogenetic protein products that will or may be available for clinical use. SUMMARY OF BACKGROUND DATA: **Bone** morphogenetic proteins comprise the osteoinductive component of several **tissue** engineering products in late-stage development as replacements for autogenous **bone** graft, and for **bone** augmentation and **repair**. METHODS: The literature on **bone** morphogenetic proteins was reviewed. RESULTS: **Bone** morphogenetic proteins were discovered originally on the basis of their presence in osteoinductive extracts of **bone matrix**. Molecular cloning of **bone** morphogenetic proteins demonstrated that they are a family of related differentiation factors, each capable of inducing the formation of new **bone tissue** when implanted. Two of the molecules in clinical use, recombinant human **bone** morphogenetic protein-2 and recombinant human **bone** morphogenetic protein-7 (OP-1) are produced in a biotechnology process using recombinant deoxyribonucleic acid technology that offers unlimited supply and substantial control over purity and reproducible activity. A third material, bovine **bone** morphogenetic protein extract, is extracted from **bone**, and contains a mixture of **bone** morphogenetic protein molecules. Each of these molecules, although

osteoinductive in vivo, has different physiologic roles and biologic activities in vivo and in vitro. Successful development of a product for use in spinal fusion involves selecting the osteoinductive molecule, the amount of the **bone** morphogenetic protein required, and the method of delivery, as well as conducting subsequent preclinical and clinical studies to evaluate its efficacy and safety. CONCLUSIONS: On the basis of the data provided in this issue of Spine, some of these **bone** morphogenetic protein-based products provide for revolutionary therapies in orthopedic practice.

6/3,AB/2 (Item 2 from file: 155)
DIALOG(R)File 155:MEDLINE(R)

13367615 22091597 PMID: 12096640
Recent advances in **tissue** engineering: an invited **review**.
Pearson R G; Bhandari R; Quirk R A; Shakesheff K M
Tissue Engineering Group, School of Pharmaceutical Sciences, University of Nottingham, NG7 2RD, UK.
Journal of long-term effects of medical implants (United States) **2002**, 12 (1) p1-33, ISSN 1050-6934 Journal Code: 9110830
Document type: Journal Article; Review; Review, Academic
Languages: ENGLISH
Main Citation Owner: NLM
Record type: Completed
Tissue formation within the body, as part of a development or **repair** process, is a complex event in which cell populations self-assemble into functional units. There is intense academic, medical, and commercial interest in finding methods of replicating these events outside the body. This interest has accelerated with the demonstration of the engineering of skin and cartilage **tissue** in the laboratory and there is now worldwide activity in the in vitro regeneration of tissues including nerve, liver, **bone**, heart valves, blood vessels, bladder, and kidney. Approaches to **tissue** engineering center on the need to provide signals to cell populations to promote cell proliferation and differentiation. This **review** considers recent advances in methods of providing these signals to cells using examples of progress in the engineering of complex tissues.

6/3,AB/3 (Item 3 from file: 155)
DIALOG(R)File 155:MEDLINE(R)

11305173 21360711 PMID: 11467074
Targets for steroid hormone mediated actions of periodontal pathogens, cytokines and therapeutic agents: some implications on **tissue** turnover in the periodontium.
Soory M
Division of Periodontology, Guy's King's and St. Thomas' Dental Institute, King's Campus, Caldecot Road, London SE5 9RW, United Kingdom. menasoory@netscapeonline.co.uk
Current drug targets (Netherlands) Dec **2000**, 1 (4) p309-25, ISSN 1389-4501 Journal Code: 100960531
Document type: Journal Article; Review; Review, Academic
Languages: ENGLISH
Main Citation Owner: NLM
Record type: Completed
This **review** attempts to encapsulate the relevance of steroid hormone action in the periodontal tissues, during inflammation, **repair** and in response to current treatment modalities. Periodontal pathogens metabolise steroid hormones which could contribute to their nutritional requirements and host evasion mechanisms, by forming capsular

proteins; their culture supernatants stimulate the synthesis of physiologically active steroid hormones by fibroblasts, which aid inflammatory **repair** . The functions of glucocorticoids, androgens, oestrogen and progesterone on connective **tissue** and **bone**, are applicable to the periodontium, being target **tissue**. This results in physiological effects on these tissues, during puberty, the menstrual cycle, pregnancy and the menopause. The effects of oral contraceptives and hormone replacement therapy on the periodontium have focused interest in the relationship between sex steroid hormones and periodontal health. Receptor expression and the role of the specific enzyme inhibitors, such as the anti-androgen finasteride and the anti-oestrogen tamoxifen, confirm target **tissue** activity for steroid hormones in the periodontium. The pro-anabolic and anti-inflammatory actions of tetracyclines, are an intriguing model for hormone mediated pathways of action. The effects of the specific alkaline phosphatase inhibitor levamisole on **matrix** turnover are linked to steroid hormone action, with direct implications on the healing periodontium. Drugs which contribute to gingival overgrowth are an interesting model, for explanation of an exaggerated 'scar **tissue**' response mediated by hormones, cytokines and a variety of enzyme systems. Cell dynamics of the periodontium plays an important role in co-ordinating the diverse interactions between steroid hormones and therapeutic agents.

6/3,AB/4 (Item 4 from file: 155)
DIALOG(R)File 155:MEDLINE(R)

11180497 21194863 PMID: 11298186
Osteopontin: a key cytokine in cell-mediated and granulomatous inflammation.
O'Regan A; Berman J S
The Pulmonary Center, Pulmonary and Critical Care Section, Boston University School of Medicine, USA.
International journal of experimental pathology (England) Dec **2000**, 81 (6) p373-90, ISSN 0959-9673 Journal Code: 9014042
Contract/Grant No.: HL04343; HL; NHLBI; HL63339; HL; NHLBI; P50-HL56386; HL; NHLBI
Document type: Journal Article; Review; Review, Academic
Languages: ENGLISH
Main Citation Owner: NLM
Record type: Completed
Osteopontin (Opn) is a secreted adhesive, glycosylated phosphoprotein that contains the arginine-glycine-aspartic acid (RGD) cell-binding sequence that is found in many extracellular **matrix** (ECM) proteins (for a **review** of Opn see References Denhardt & Guo 1993; Patarca et al. 1993; Rittling & Denhardt 1999). Since its initial description in 1979 as a secreted protein associated with malignant transformation, Opn has been independently discovered by investigators from diverse scientific disciplines, and has been associated with a remarkable range of pathologic responses. Opn is an important **bone matrix** protein, where it is thought to mediate adhesion of osteoclasts to resorbing **bone**. However, studies from the past decade have identified an alternative role for Opn as a key cytokine regulating **tissue repair** and inflammation. Recent work by our laboratory and that of others has underlined the importance of Opn as a pivotal cytokine in the cellular immune response. Despite this Opn is not well known to the immunologist. In this **review** we will focus on studies that pertain to the role of Opn in cell-mediated and granulomatous inflammation.

6/3,AB/5 (Item 5 from file: 155)
DIALOG(R)File 155:MEDLINE(R)

11059247 21062465 PMID: 11094454
Emerging strategies of **bone** and joint **repair**.
Schultz O; Sittinger M; Haeupl T; Burmester G R
Department of Rheumatology and Immunology, Charite, Humboldt-University of Berlin, Berlin, Germany.
Arthritis research England 2000, 2 (6) p433-6, ISSN 1465-9905 Journal Code: 100913255
Document type: Journal Article; Review; Review, Tutorial
Languages: ENGLISH
Main Citation Owner: NLM
Record type: Completed
The advances in biomedicine over the past decade have provided revolutionary insights into molecules that mediate cell proliferation and differentiation. Findings on the complex interplay of cells, growth factors, **matrix** molecules and cell adhesion molecules in the process of **tissue** patterning have vitalized the revolutionary approach of bioregenerative medicine and **tissue** engineering. Here we **review** the impact of recent work in this interdisciplinary field on the treatment of musculoskeletal disorders. This novel concept combines the transplantation of pluripotent stem cells, and the use of specifically tailored biomaterials, arrays of bioactive molecules and gene transfer technologies to direct the regeneration of pathologically altered musculoskeletal tissues.

6/3,AB/6 (Item 6 from file: 155)
DIALOG(R)File 155:MEDLINE(R)

10907870 20455165 PMID: 11001527
Tissue engineering via local gene delivery.
Bonadio J
Selective Genetics, Inc., San Diego, CA 92121, USA. jbonadio@earthlink.net
Journal of molecular medicine (Berlin, Germany) (GERMANY) 2000, 78 (6) p303-11, ISSN 0946-2716 Journal Code: 9504370
Document type: Journal Article; Review; Review, Tutorial
Languages: ENGLISH
Main Citation Owner: NLM
Record type: Completed
The first goal of this **review** is to describe a local plasmid gene transfer technology known as the gene activated **matrix** (GAM). GAM was the first gene therapy designed specifically for **tissue** engineering applications, and the mechanism of action of plasmid gene transfer is closely tied to the normal sequence of events associated with wound healing. The normal sequence of wound healing events is stereotyped for most tissues, and one assumption has been that GAM could serve as a platform technology for local gene delivery in various tissues and organs. This hypothesis essentially has been proved: animal studies over the past 6 years have established that plasmid genes can be delivered to acutely injured tendon, ligament, **bone**, muscle, skin, and nerve. The second goal of the **review** is to describe the most likely "first use" of the technology in man, namely, treatment of osteoporotic hip fracture in the elderly. Although not universally appreciated, interest in osteoporotic fracture should grow because of epidemiological, surgical, and societal considerations. These considerations, plus the unmet clinical need associated with the current standard of fracture care, justify efforts to develop novel therapies for **bone** regeneration and **repair** in the elderly.

6/3,AB/7 (Item 1 from file: 5)
DIALOG(R)File 5:Biosis Previews(R)

14030173 BIOSIS NO.: 200300024202
Glucose transport and metabolism in chondrocytes: A key to understanding chondrogenesis, skeletal development and cartilage degradation in osteoarthritis.
AUTHOR: Mobasheri A a ; Vannucci S J; Bondy C A; Carter S D; Innes J F; Arteaga M F; Trujillo E; Ferraz I; Shakibaei M; Martin-Vasallo P
AUTHOR ADDRESS: a Department of Veterinary Preclinical Sciences, Faculty of Veterinary Science, University of Liverpool, Liverpool, L69 7ZJ, UK**
UK E-Mail: a.mobasheri@liverpool.ac.uk
JOURNAL: Histology and Histopathology 17 (4) :p1239-1267 October 2002
2002
MEDIUM: print
ISSN: 0213-3911
DOCUMENT TYPE: Literature Review
RECORD TYPE: Abstract
LANGUAGE: English

ABSTRACT: Despite the recognition that degenerative cartilage disorders like osteoarthritis (OA) and osteochondritis dissecans (OCD) may have nutritional abnormalities at the root of their pathogenesis, balanced dietary supplementation programs have played a secondary role in their management. This **review** emphasizes the importance and role of nutritional factors such as glucose and glucose-derived sugars (i.e. glucosamine sulfate and vitamin C) in the development, maintenance, **repair**, and remodeling of cartilage. Chondrocytes, the cells of cartilage, consume glucose as a primary substrate for ATP production in glycolysis and utilize glucosamine sulfate and other sulfated sugars as structural components for extracellular **matrix** synthesis and are dependant on hexose uptake and delivery to metabolic and biosynthetic pools. Data from several laboratories suggests that chondrocytes express multiple isoforms of the GLUT/SLC2A family of glucose/polyol transporters. These facilitative glucose transporter proteins are expressed in a **tissue** and cell-specific manner, exhibit distinct kinetic properties, and are developmentally regulated. They may also be regulated by endocrine factors like insulin and insulin-like growth factor I (IGF-I) and cytokines such as interleukin 1 beta (IL-1beta) and tumour necrosis factor alpha (TNF-alpha). Recent studies suggest that degeneration of cartilage may be triggered by metabolic disorders of glucose balance and that OA occurs coincident with metabolic disease, endocrine dysfunction and diabetes mellitus. Based on these metabolic, endocrine and developmental considerations we present a novel hypothesis regarding the role of glucose transport and metabolism in cartilage physiology and pathophysiology and speculate that supplementation with sugar-derived vitamins and nutraceuticals may benefit patients with degenerative joint disorders.

2002

6/3,AB/8 (Item 1 from file: 444)
DIALOG(R)File 444:New England Journal of Med.

00122833

Medical Progress: IgA Nephropathy (**Review** Article)

Donadio, James V.; Grande, Joseph P.
The New England Journal of Medicine
Sep 5, **2002**; 347 (10),pp 738-748
LINE COUNT: 00552 WORD COUNT: 07618

6/3,AB/9   (Item 2 from file: 444)
DIALOG(R)File 444:New England Journal of Med.

00121016

Advances in Immunology: Allergy and Allergic Diseases First of Two Parts (**Review** Articles)

Kay, A.B.
The New England Journal of Medicine
Jan 4, **2001**; 344 (1),pp 30-37
LINE COUNT: 00371   WORD COUNT: 05125

6/3,AB/10   (Item 3 from file: 444)
DIALOG(R)File 444:New England Journal of Med.

00120247

Mechanisms of Disease: Role of Transforming Growth Factor (beta) in Human Disease (**Review** Articles)

Blobe, Gerard C.; Schiemann, William P.; Lodish, Harvey F.
The New England Journal of Medicine
May 4, **2000**; 342 (18),pp 1350-1358
LINE COUNT: 00583   WORD COUNT: 08057

6/3,AB/11   (Item 4 from file: 444)
DIALOG(R)File 444:New England Journal of Med.

00120246

Medical Progress: The Acute Respiratory Distress Syndrome (**Review** Articles)

Ware, Lorraine B.; Matthay, Michael A.
The New England Journal of Medicine
May 4, **2000**; 342 (18),pp 1334-1349
LINE COUNT: 00684   WORD COUNT: 09444

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9/3,AB/1   (Item 1 from file: 155)
DIALOG(R)File 155:MEDLINE(R)

14500908   22493267   PMID: 12603699
Bone marrow stem cells regenerate infarcted myocardium.
Orlic Donald; Kajstura Jan; Chimenti Stefano; Bodine David M; Leri Annarosa; Anversa Piero
Genetics and Molecular Biology Branch, NHGRI, NIH, Bethesda, MD 20892, Department of Medicine, New York Medical College, Valhalla, NY 10595, USA.
Pediatric transplantation (Denmark) Apr **2003**, 7 (3 Suppl) p86-8, ISSN 1397-3142 Journal Code: 9802574
Document type: Journal Article
Languages: ENGLISH
Main Citation Owner: NLM
Record type: In Process
Heart disease is the leading cause of death in the United States for both men and women. Nearly 50% of all cardiovascular deaths result from coronary artery disease. Occlusion of the left coronary artery leads to ischemia, infarction, necrosis of the affected myocardial **tissue** followed by scar formation and loss of function. Although myocytes in the surviving myocardium undergo hypertrophy and cell division occurs in the border area of the dead **tissue** , myocardial infarcts do not regenerate and eventually result in the death of the individual. Numerous attempts have been made to repair damaged myocardium in animal models and in humans. **Bone marrow** stem cells (BMSC) retain the ability throughout adult life to self-renew and differentiate into cells of all **blood** lineages. These adult BMSC have recently been shown to have the capacity to differentiate into multiple specific cell types in tissues other than **bone marrow**. Our research is focused on the capacity of BMSC to form new cardiac myocytes and coronary vessels following an induced myocardial infarct in adult mice. In this paper we will **review** the data we have previously published from studies on the regenerative capacity of BMSC in acute ischemic myocardial injury. In one experiment donor BMSC were injected directly into the healthy myocardium adjacent to the injured area of the left ventricle. In the second experiment, mice were treated with cytokines to mobilize their BMSC into the circulation on the theory that the stem cells would traffic to the myocardial infarct. In both

experimental protocols, the BMSC gave rise to new cardiac myocytes and coronary **blood** vessels. This BMSC-derived myocardial regeneration resulted in improved cardiac function and survival.

9/3,AB/2 Item 2 from file: 155
DIALOG(R)File 155:MEDLINE(R)

14413193 22455241 PMID: 12568297
Genetic control of stem cells: implications for aging.
Van Zant Gary
Department of Internal Medicine, Markey Cancer Center, Division of Hematology/Oncology, University of Kentucky, Lexington, Kentucky 40536-0093, USA. gvzant1@uky.edu
International journal of hematology (Ireland) Jan **2003**, 77 (1) p29-36, ISSN 0925-5710 Journal Code: 9111627
Document type: Journal Article
Languages: ENGLISH
Main Citation Owner: NLM
Record type: In Process
Stem cells are currently at the center of both controversy and notoriety. The harvest of human embryonic or fetal stem cells, at least with methods available now, necessarily involves the sacrifice of the embryo or fetus. This critical step in the procurement of stem cells has stimulated intense discussion at all levels of academia, government, and society in general. What societal benefits, if any, justify such a strategy for obtaining these stem cells? In other species it has been possible to generate virtually all cell types found in adult organs from embryonic stem cells. This ability has opened endless clinical possibilities for **tissue** and organ replacement through the transplantation of cells derived from embryonic stem cells. Luckily, there may be an alternative to this ethical dilemma. It is becoming increasingly clear that stem cells exist in many, if not all, adult tissues. Adult stem cells normally replenish **tissue** cells lost through the wear and tear of aging or damage from injury or disease. With the proper coaxing in **tissue** culture and when transplanted, these stem cells may regenerate the full repertoire of organotypic cells and thus may therapeutically regenerate tissues in vivo in much the same way as embryonic stem cells do. For several reasons, the best-studied stem cells are those of the **blood**-forming system. Mature **blood** cells generally have short functional life spans, usually measured in days, and therefore require replenishment at a steady pace throughout one's lifetime. Stem cells are intimately involved in this renewal and, because of the relative ease of access to the **bone marrow**, stem cells have been well studied. Second, **bone marrow** transplantation following radiation or high-dose chemotherapy in the treatment of cancer has fostered research on the basic biology and therapeutic uses of hematopoietic stem cells over the more than 30 years stem cell transplantation has been used clinically. It is my aim to **review** what is known about the genes controlling hematopoietic stem cell function. Identifying, and ultimately manipulating, the genes that regulate stem cell number, replication rate, and self-renewal capacity may have important clinical benefits. I discuss evidence suggesting that the characterization of least some of these stem cell genes will shed light on mechanisms important in the aging process. I advance the hypothesis that stem cells accumulate cellular damage during aging that diminishes their developmental potency and ability to replenish **blood** cells, particularly after hematopoietic stress. In this view, the impaired function of stem cells in hematopoietic and in other self-renewing tissues limits the longevity of animals, and perhaps of humans.

9/3,AB/3 Item 3 from file: 155
DIALOG(R)File 155:MEDLINE(R)

14347983 22356404 PMID: 12468917
Hepatic venoocclusive disease in **blood** and **bone marrow** transplantation in children and young adults: incidence, risk factors, and outcome in a cohort of 241 patients.
Reiss Ulrike; Cowan Morton; McMillan Alex; Horn Biljana; et al
Department of Hematology Oncology, Children's Hospital Oakland, Oakland, CA, USA.
Journal of pediatric hematology/oncology - official journal of the American Society of Pediatric Hematology/Oncology (United States) Dec **2002**, 24 (9) p746-50, ISSN 1077-4114 Journal Code: 9505928
Comment in J Pediatr Hematol Oncol. 2002 Dec;24(9) 706-9; Comment in PMID 12468908
Document type: Journal Article
Languages: ENGLISH
Main Citation Owner: NLM
Record type: Completed
PURPOSE: To describe recent characteristics of incidence, risk factors, treatment, and outcome of venoocclusive disease (VOD) in children and young adults undergoing **blood** and **bone marrow** transplantation (BMT). METHODS: All children and young adults (n = 241) undergoing first myeloablative transplant at the UCSF Pediatric BMT unit between 1992 and 2000 were included. Retrospective chart **review** was done. Descriptive statistics and univariate and multivariate analyses of risk factors are presented. RESULTS: Venoocclusive disease developed in 65 patients (27%); it was severe in 13/65 patients (20%). Matched unrelated donor transplantation, advanced-stage malignancies, and transplantation in the recent period (1998-2000) were identified as significant risk factors for VOD in univariate and multivariate analyses. Heparin prophylaxis did not decrease the incidence of VOD. Venoocclusive disease was diagnosed at a median day 8 after BMT. Five of 13 patients with severe VOD (38%) survived for more than 1 year after BMT, even after renal and respiratory failure and high total bilirubin levels up to 35 mg/dL. Nine of the 13 patients received fibrinolytic treatment with **tissue** plasminogen activator, anti-thrombin 3, or defibrotide. The survival rate at day 100 after BMT for children with VOD was 77%; it was 94% for those without VOD. CONCLUSIONS: The persistently high incidence of VOD, its significant impact on posttransplant survival, and the demonstration of recovery from even severe VOD underscore the importance of early diagnosis and initiation of specific therapy. The use of Bearman's model of prediction of severity of VOD and the application of fibrinolytic drugs when adequate are highly recommended.

9/3,AB/4 (Item 4 from file: 155)
DIALOG(R)File 155:MEDLINE(R)

14239463 22015402 PMID: 12021492
Neural stem cells: plasticity and their transdifferentiation potential.
Vescovi Angelo; Gritti Angela; Cossu Giulio; Galli Rossella; et al
Stem Cell Research Institute, DIBIT H. San Raffaele, Milan, Italy. vescovi.angelo@hsr.it
Cells, tissues, organs (Switzerland) **2002**, 171 (1) p64-76, ISSN 1422-6405 Journal Code: 100883360
Document type: Journal Article; Review; Review, Tutorial
Languages: ENGLISH
Main Citation Owner: NLM
Record type: Completed
The presence of resident stem cells in adult tissues is of fundamental importance for the maintenance of their structural and functional integrity. In fact, throughout life, somatic stem cells attend to the critical function of substituting terminally differentiated cells lost to physiological turnover, injury or disease. Thence, one of the basic dogmata

in **tissue** biology holds that the differentiation potential of an adult stem cell is restricted to the generation of the mature cell lineages found in the **tissue** to which the stem cell belongs. A plethora of recent evidences from many groups, including ours, is now providing evidence that adult stem cells may possess a broader differentiation repertoire than expected and that their fate potential may not be as **tissue** specific as once thought. The initial example of an unforeseen, trans-germ layer plasticity - that seems now to emerge as a prototypic functional trait of various somatic stem cells of different origin - has come from the reported awakening of a latent hemopoietic developmental capacity in stem cells isolated from the adult mammalian brain following their transplantation into sub-lethally irradiated mice. More recently, it has been shown that adult neural stem cells can differentiate into a wide array of bodily cells of different origin when injected into the blastocyst and into myogenic cells when transplanted into the adult regenerating skeletal muscle. Moreover, **bone marrow** stem cells can now give rise to skeletal muscle, hepatic and brain cells, whereas muscle precursors can generate **blood** cells. In this article, we **review** some of the basic notions regarding the functional properties of the adult neural stem cells and discuss findings in the expanding area of trans-germ layer conversion, with emphasis on the neural stem cell.

9/3,AB/5 (Item 5 from file: 155)
DIALOG(R)File 155:MEDLINE(R)

14233820 22395990 PMID: 12508164
[In search of renal stem cells]
Alla ricerca della cellula staminale renale.
Anglani F; Forino M; Gambaro G; D'Angelo A; et al
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Giornale italiano di nefrologia - organo ufficiale della Societa italiana di nefrologia (Italy) Nov-Dec **2002**, 19 (6) p607-16, ISSN 0393-5590 Journal Code: 9426434
Document type: Journal Article
Languages: ITALIAN
Main Citation Owner: NLM
Record type: In Process
The therapeutic potential of stem cell research is very promising. Although arising ethical questions, especially in the field of embryonic stem cells (ES), it is astonishing how, in the last few years, the potential application of stem cells for treating proliferative as well as degenerative diseases, is becoming increasingly evident. It was recently demonstrated that somatic stem cells showed unexpected plasticity similar to ES. In fact, if somatic stem cells are exposed to proper stimuli they can differentiate into a multitude of cell types that may be different from those of the **tissue** they belong to. In addition, it was recently demonstrated that circulating **blood** stem cells, probably of **bone marrow** origin, were recruited at the sites of injury to regenerate or repair damaged tissues. Very little is known about renal stem cells. Although the great capacity of the kidney to regenerate injured nephrons is well established, renal somatic stem cells have yet to be identified. The question we are now faced with is whether renal stem cells exist and, if they do exist, where do they reside. In the attempt to answer this question, the present **review** will focus on the achievements both in the fields of somatic stem cells and renal embryogenesis and in the field of renal repair and regeneration mechanisms.

9/3,AB/6 (Item 6 from file: 155)
DIALOG(R)File 155:MEDLINE(R)

14224078 22390506 PMID: 12502965
Bone marrow biopsy: interpretive guidelines for the surgical pathologist.
Cotelingam James D; et al
Advances in anatomic pathology United States Jan **2003**, 10 (1) p8-26, ISSN 1072-4109 Journal Code: 9435676
Document type: Journal Article
Languages: ENGLISH
Main Citation Owner: NLM
Record type: In Process
Ideally, the **bone marrow** core biopsy should be reviewed with knowledge of the clinical history, complete **blood** count, and findings in the peripheral **blood** and **bone marrow** aspirate smears. However, for a variety of reasons, the pathologist may receive the core biopsy and aspirate clot section without all of this information. Although this approach is not optimal, a great deal of valuable information can be generated from these specimens. Over the past 20 years, there has been considerable progress in the fields of flow cytometric analysis, immunohistochemistry, and molecular diagnostic studies that can be performed on smears or extracted DNA from paraffin embedded **tissue**. These modalities have augmented and refined diagnostic criteria formerly ascertained by light microscopy, cytochemistry, and cytogenetics. This is particularly true of some myeloid and lymphoreticular neoplasms where a collaborative and multidisciplinary approach to the diagnosis has become necessary. Despite this growing complexity and dependence on newer methodologies, the traditional role of histopathology in evaluating the **bone marrow** biopsy remains as important as it has been in the past. In this **review** , we focus on contemporary practices and expectations for interpreting **bone marrow** biopsies and clot sections.

9/3,AB/7 (Item 7 from file: 155)
DIALOG(R)File 155:MEDLINE(R)

14078991 22053270 PMID: 12056776
Clinicopathological and immunophenotypical features of canine intravascular lymphoma (malignant angioendotheliomatosis).
McDonough S P; Van Winkle T J; Valentine B A; vanGessel Y A; Summers B A; et al
Department of Biomedical Sciences, College of Veterinary Medicine, Cornell University, Upper Tower Road, Ithaca, NY14853-6401, USA.
Journal of comparative pathology (England) May **2002**, 126 (4) p277-88, ISSN 0021-9975 Journal Code: 0102444
Document type: Journal Article
Languages: ENGLISH
Main Citation Owner: NLM
Record type: Completed
Intravascular lymphoma (IVL) is a rare angiotropic large-cell lymphoma in which neoplastic lymphocytes proliferate within the lumina of **blood** vessels in the absence of a primary extravascular mass or leukaemia. A retrospective **review** of veterinary medical records identified 17 cases of canine IVL. Spinal cord ataxia (seven dogs), posterior paralysis (one dog), seizures (four dogs) and vestibular disease (three dogs) dominated the clinical presentation. Haemorrhage, ischaemia, and occasional foci of vascular proliferation were found in **tissue** sections from affected dogs. Vessels, predominantly veins, throughout the body were frequently filled with neoplastic lymphocytes. Splenic involvement occurred in only one of 10 cases examined and **bone marrow** involvement was absent in four cases examined. Formalin-fixed paraffin wax-embedded tissues from 15 cases were examined immunohistochemically with

streptavidin-biotin-horseradish peroxidase and a catalysed signal amplification system. The neoplastic cells were classified in eight cases as T cells (CD3+ IgG- CD79a-), in one case as B cells (CD3- CD79a.dim IgG+), and in the remaining six cases as non-T, non-B (CD3- IgG- CD79a-). The clinical and pathological features of canine IVL closely resembled those of the human disease. In striking contrast to human cases, which are most often B-cell lymphomas, the immunophenotypes of the canine IVLs in this series were heterogeneous. The canine IVLs were derived primarily from T cells and non-T, non-B lymphocytes, B cells being found in only a single instance.

9/3,AB/8 (Item 8 from file: 155)
DIALOG(R)File 155:MEDLINE(R)

13988343 22265642 PMID: 12377929
The mononuclear phagocyte system revisited.
Hume David A; Ross Ian L; Himes S Roy; Sasmono P Tedjo; Wells Christine A; Ravasi Timothy; et al
Institute for Molecular Bioscience, University of Queensland, Australia.
D.Hume@imb.uq.edu.au
Journal of leukocyte biology (United States) Oct **2002**, 72 (4) p621-7, ISSN 0741-5400 Journal Code: 8405628
Document type: Journal Article; Review; Review, Tutorial
Languages: ENGLISH
Main Citation Owner: NLM
Record type: Completed
The mononuclear phagocyte system (MPS) was defined as a family of cells comprising **bone marrow** progenitors, **blood** monocytes, and **tissue** macrophages. In this **review**, we briefly consider markers for cells of this lineage in the mouse, especially the F4/80 surface antigen and the receptor for macrophage colony-stimulating factor. The concept of the MPS is challenged by evidence that there is a separate embryonic phagocyte lineage, the blurring of the boundaries between macrophages and other cells types arising from phenotypic plasticity and transdifferentiation, and evidence of local renewal of **tissue** macrophage populations as opposed to monocyte recruitment. Nevertheless, there is a unity to cells of the MPS suggested by their location, morphology, and shared markers. We discuss the origins of macrophage heterogeneity and argue that macrophages and antigen-representing dendritic cells are closely related and part of the MPS.

9/3,AB/9 (Item 9 from file: 155)
DIALOG(R)File 155:MEDLINE(R)

13961872 22226502 PMID: 12241534
Adult stem cell plasticity: new pathways of **tissue** regeneration become visible.
Forbes Stuart J; Vig Pamela; Poulsom Richard; Wright Nicholas A; Alison Malcolm R; et al
Histopathology Unit, Cancer Research UK, London, U.K. and Department of Medicine, Faculty of Medicine, Imperial College of Science, Technology and Medicine (ICSTM), St Mary's Hospital, London, U.K.
Clinical science (London, England - 1979) (England) Oct **2002**, 103 (4) p355-69, ISSN 0143-5221 Journal Code: 7905731
Document type: Journal Article
Languages: ENGLISH
Main Citation Owner: NLM
Record type: In Process
There has recently been a significant change in the way we think about

organ regeneration. In the adult, organ formation and regeneration was thought to occur through the action of organ-or **tissue**-restricted stem cells i.e. haematopoietic stem cells making **blood**; gut stem cells making gut, etc. . However, there is a large body of recent work that has extended this model. Thanks to lineage tracking techniques, we now believe that stem cells from one organ system, for example the haematopoietic compartment, can develop into the differentiated cells within another organ system, such as liver, brain or kidney. This cellular plasticity not only occurs under experimental conditions, but has also been shown to take place in humans following **bone marrow** and organ transplants. This trafficking is potentially bi-directional, and even differentiated cells from different organ systems can interchange, with pancreatic cells able to form hepatocytes, for example. In this **review** we will detail some of these findings and attempt to explain their biological significance.

9/3,AB/10 (Item 10 from file: 155)
DIALOG(R)File 155:MEDLINE(R)

13949799 22205105 PMID: 12216947
Platelet antigens. The role of human platelet alloantigens (HPA) in **blood** transfusion and transplantation.
Rozman Primoz; et al
Blood Transfusion Centre of Slovenia, Department of Immunohematology, Ljubljana.
Transplant immunology (England) Aug **2002**, 10 (2-3) p165-81,
ISSN 0966-3274 Journal Code: 9309923
Document type: Journal Article
Languages: ENGLISH
Main Citation Owner: NLM
Record type: In Process
In this **review** , we describe the platelet surface molecules with special focus on the polymorphic glycoproteins giving rise to the human platelet alloantigen (HPA) system. We list the platelet glycoprotein complexes GPIa/IIa, GPIIb/IIIa, GPIb/V/IX and some other molecules, the corresponding genes that encode them and we describe their polymorphisms as well as their physiological function. Based on data obtained by serological and molecular methods, we explain how ancestral HPA alloepitopes have developed into the modern variants. We also describe the **tissue** distribution of these proteins, which seems to be wider than thought initially, and discuss the significance of the HPA encoding genes distribution in various populations. Methods for their determination are then described briefly Since HPA alloepitopes can induce antibodies with resulting clinical conditions such as: post-transfusion refractoriness to platelets (PTR); post-transfusion thrombocytopenic purpura (PTTP); and fetomaternal alloimmune thrombocytopenia (FMAIT), the mechanism of this alloimmunization and its prevention is described. Although the humoral arm is more important from the clinical viewpoint, the activation of the cytotoxic arm by HPA alloepitopes is also possible. These polymorphisms also seem to have a role in certain other clinical circumstances, therefore their impact on cardiovascular diseases and haemostatic disorders as well as their role in the transplantation of solid organs and **bone marrow** is addressed. We conclude that during the last decades, the research of the platelet membrane molecules contributed considerably to the diagnostics, prevention and therapy of the **blood** coagulation and haemostatic disorders, to the management of the neonatal thrombocytopenias, transfusion-related thrombocytopenias, refractoriness to platelet transfusions and autoimmune disorders. It also changed our view on the role of HPA alloepitopes in **bone marrow** and solid organ transplantation. In the future, this accumulated knowledge will be useful for the development of the cell based therapies and immune modulation of both acquired and hereditary diseases.

13896246   22041782   PMID: 12046856

Stem cell plasticity and **blood** and **marrow** transplantation: a clinical strategy.

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Journal of cellular biochemistry. Supplement (United States) **2002**, 38 p96-103, ISSN 0733-1959 Journal Code: 8207539
Document type: Journal Article; Review; Review, Tutorial
Languages: ENGLISH
Main Citation Owner: NLM
Record type: Completed

The newly described phenomenon of stem cell plasticity raises interesting biological questions and offers exciting opportunities in clinical application. This **review** uses the well-established practice of **blood** and **marrow** transplantation as a paradigm to explore the clinical consequences of this finding. Recently proposed non-myeloablative conditioning regimens have shown that mixed donor-host hematolymphoid chimerism can be established with relatively low toxicity in both animal studies and human trials. Hematopoietic growth factor treatment of transplanted patients can mobilize a large number of donor stem cells to migrate from **marrow** to non-hematopoietic organs. We propose that these advances, in conjunction with the developmental plasticity of stem cells, can constitute components of a clinical strategy to use **blood** and **marrow** transplantation as a platform to treat systemic diseases involving non-hematopoietic tissues.

13754837   22194576   PMID: 12205681

Neural cells derived from adult **bone marrow** and umbilical cord **blood**.

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Center of Aging and Brain Repair, University of South Florida and James Haley VA Hospital Health Science Center, Tampa, Florida 33612, USA.
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Journal of neuroscience research (United States) Sep 15 **2002**, 69 (6) p880-93, ISSN 0360-4012 Journal Code: 7600111
Document type: Journal Article; Review; Review Literature
Languages: ENGLISH
Main Citation Owner: NLM
Record type: Completed

Under experimental conditions, **tissue**-specific stem cells have been shown to give rise to cell lineages not normally found in the organ or **tissue** of residence. Neural stem cells from fetal brain have been shown to give rise to **blood** cell lines and conversely, **bone marrow** stromal cells have been reported to generate skeletal and cardiac muscle, oval hepatocytes, as well as glia and neuron-like cells. This article reviews studies in which cells from postnatal **bone marrow** or umbilical cord **blood** were induced to proliferate and differentiate into glia and neurons, cellular lineages that are not their normal destiny. The **review** encompasses in vitro and in vivo studies with focus on experimental variables, such as the source and characterization of cells, cell-tracking methods, and markers of neural

differentiation. The existence of stem progenitor cells with previously unappreciated proliferation and differentiation potential in postnatal **bone marrow** and in umbilical cord **blood** opens up the possibility of using stem cells found in these tissues to treat degenerative, post-traumatic and hereditary diseases of the central nervous system.

9/3,AB/13 Item 13 from file: 155
DIALOG(R)File 155:MEDLINE(R)

13572792 21952942 PMID: 11956600

A summary of two clinical studies on tumor cell dissemination in primary and metastatic breast cancer: methods, prognostic significance and implication for alternative treatment protocols (**Review**).

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International journal of oncology (Greece) May **2002**, 20 (5) p1027-34, ISSN 1019-6439 Journal Code: 9306042

Document type: Journal Article; Review; Review, Tutorial

Languages: ENGLISH

Main Citation Owner: NLM

Record type: Completed

Although only less than 10% of women with primary breast cancer have clinicopathologic signs of overt metastases, metastatic relapse occurs in about half of the cases with apparently localized tumors within five years after surgery. In 23% of the patients, **bone marrow** metastases are detectable at first relapse and this rate even increases in patients with metastatic breast cancer. However, hematogeneous or lymphatic spread of occult tumor cells can arise before diagnosis at an early stage of primary tumor growth and is regularly underestimated by currently available clinical and pathologic staging procedures. We studied cytokeratin-positive (CK+) cells in the **bone marrow** (BM) and tumor markers in the **blood** of 128 patients with primary breast cancer in order to obtain an early diagnosis of residual disease. In a second study, we monitored cytokeratin (CK)/17-1A positive cells in the BM and peripheral **blood** stem cells (PBSC) to evaluate whether dose intensive or high-dose (HD)-chemotherapy can eliminate micrometastases in high-risk breast cancer patients. The overall CK+ rate was 34% (44/128 patients), 29% (15/51) for patients with T1 tumors, 33% (28/84) for N0 patients and 31% (26/82) for patients with G1-2 breast carcinoma. Interestingly, 67% of CK+ patients were only positive in one of the two BM aspirates studied. At least one tumor marker including carcinoembryonic antigen, carbohydrate antigen 15-3 and **tissue** polypeptide antigen, was increased in 58/128 (45%) patients [21/58 (36%) were CK+ in the BM]. Surprisingly, levels for the extracellular domain of Her-2/neu in serum samples were within the normal range in every patient studied. After a 2-year follow-up, 7/128 patients relapsed (3/7 CK+/TM-; 2/7 CK-/TM+; 2/7 CK-/TM-). We concluded that studying two BM aspirates for CK+ cells by immunocytochemistry in combination with tumor marker determination is useful for identifying patients with a higher risk for relapse. A tumor cell enrichment technique, applied in 70 patients prior to immunocytochemistry using dynabeads directly coupled to an antibody (BerEp4) targeting the 17-1A antigen, did not enhance the detection rate of disseminated tumor cells in this patient group. We monitored CK+/17-1A+ cells in the BM and PBSC and studied Her-2/neu serum levels of patients with locally advanced (n=13, group 1) and metastatic breast cancer (n=30, group 2). CK+ cells were found in the BM of 3/13 (23%) group 1 patients before but not after chemotherapy resulting in an overall survival (OS) of 92% after a median follow-up of 33 months. Contamination of PBSC in 2/9 (22%) patients was not associated with decreased survival. In group 2 patients, the CK+ rate was 60% (18/30 patients) before and 40% (4/10 patients) after therapy with an OS rate of

43% after 29 months. PBSC samples were positive in 7 24 29% patients. CK+ BM and PBSC led to a rapid progress and short OS whereas tumor cell free BM and PBSC resulted in a mean OS of 30 months. The antigen 17-1A was detected on most CK+ cells in both patient groups before therapy, on all CK+ PBSC and on CK+ cells in group 2 patients after therapy. Increased Her-2 neu levels were found in group 2 patients before chemotherapy. In conclusion, micrometastatic cells are present in **blood** and PBSC grafts of high-risk breast cancer patients and can survive even HD-chemotherapy. Immunotherapeutic target antigens on the cell surface of these cells support the idea that a combined chemoimmunotherapy might be successful in eliminating minimal residual disease.

9/3,AB/14 (Item 14 from file: 155)
DIALOG(R)File 155:MEDLINE(R)

13404248 21910377 PMID: 11907746
Radiologic placement of a low profile implantable venous access port in a pediatric population.
Nosher J L; Bodner L J; Ettinger L J; Siegel R L; Gribbin C; Asch J; Drachtman R A
Department of Radiology, UMDNJ-Robert Wood Johnson Medical School, New Brunswick, NJ 08903-0019, USA. nosher@umdnj.edu
Cardiovascular and interventional radiology (United States) Nov-Dec **2001**, 24 (6) p395-9, ISSN 0174-1551 Journal Code: 8003538
Document type: Evaluation Studies; Journal Article
Languages: ENGLISH
Main Citation Owner: NLM
Record type: Completed
PURPOSE: To evaluate the feasibility and complications of placement of a low-profile venous access port in the chest in children requiring long-term venous access. METHOD: A low-profile peripheral arm port (PAS port; Sims Deltec, St. Paul, MN, USA) was implanted in the chest in 22 children over a 4-year period. The mean age of the study group was 6 years (range: 9 months to 20 years). Ports were placed for the administration of chemotherapy, hyperalimentation and frequent **blood** sampling. Sonographic guidance was used to access the internal jugular or subclavian vein in each case. A **review** of all inpatient and outpatient charts was undertaken to assess catheter performance and complications. RESULTS: Access to the central venous circulation was successfully achieved in each case without complication. Ports remained implanted for 6579 catheter-days (mean: 299 days). Ten ports have been removed. Of three patients (13%) experiencing device-related infections (0.45 infections/1000 catheter days), two (9.1%) were unresponsive to antibiotics and removed (0.3 infections/1000 catheter days). One port was removed because of pain in the shoulder adjacent to the port implantation site. One port was removed because of difficult access. The final port was removed in order to place a dual-lumen catheter prior to **bone marrow** transplant. Twelve ports remain implanted. Aspiration occlusion occurred in four patients (18%). Deep venous thrombosis did not occur in any patient. CONCLUSION: Low-profile chest ports placed by interventional radiologists in the interventional radiology suite can be placed in children as safely as traditional chest ports placed in the operating room. The incidence of infection, venous thrombosis and aspiration occlusion is comparable to that of ports placed operatively.

9/3,AB/15 (Item 15 from file: 155)
DIALOG(R)File 155:MEDLINE(R)

13167754 21917656 PMID: 11919420
Mast cell hyperplasia: role of cytokines.
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International archives of allergy and immunology Switzerland Feb **2002**, 127 (2) p118-22, ISSN 1018-2438 Journal Code: 9211652
Document type: Journal Article; Review; Review, Tutorial
Languages: ENGLISH
Main Citation Owner: NLM
Record type: Completed
Mast cell hyperplasia is found in different pathologies such as chronic inflammatory processes, fibrotic disorders, wound healing or neoplastic **tissue** transformation. The functional significance of the accumulation of mast cells in these processes is largely unknown. It is now established that **bone marrow**-derived mast cell progenitors circulate in peripheral **blood** and subsequently migrate into the **tissue** where they undergo final maturation under the influence of local microenvironmental factors. Cytokines are of particular importance for mast cell recruitment, development, and function. Stem cell factor (SCF) is a unique mast cell growth factor, since mast cells disappear completely in the absence of SCF. However, several other cytokines such as IL-3 and IL-4 have been shown to influence mast cell proliferation and function also. This **review** focuses on the role of cytokines in the regulation of mast cell hyperplasia. Copyright 2002 S. Karger AG, Basel

9/3,AB/16 (Item 16 from file: 155)
DIALOG(R)File 155:MEDLINE(R)

13052713 21632195 PMID: 11776471
Common origins of **blood** and **blood** vessels in adults?
Hirschi K; Goodell M
Dept. of Pediatrics, Children's Nutrition Research Center, Houston, TX, USA. khirschi@bcm.tmc.edu
Differentiation; research in biological diversity (Germany) Oct **2001**, 68 (4-5) p186-92, ISSN 0301-4681 Journal Code: 0401650
Document type: Journal Article; Review; Review, Tutorial
Languages: ENGLISH
Main Citation Owner: NLM
Record type: Completed
After embryonic development, the vast majority of cells are differentiated and all organs are in place. Growth of the organism then ensues and continues until adulthood, whereupon cell division largely ceases. In some tissues, notably the **bone marrow**, skin, and gut, cell proliferation continues throughout life to replace cells lost by attrition. This regeneration is fueled by rare, long-lived, and largely quiescent stem cells that give rise to committed progenitors, which in turn generate large numbers of fully differentiated cells. Mounting evidence suggests that such cells can significantly contribute to **tissue** repair and regeneration in adults and may therefore prove beneficial for autologous cell and gene therapies. This **review** focuses on the potential of adult stem cells to give rise to hematopoietic and vascular cells. We discuss evidence that a highly purified population of adult stem cells, termed SP cells, serves as a hematopoietic progenitor and can contribute to vascular regeneration after injury. We also discuss the potential relationship of these cells to the embryonic hemangioblast.

9/3,AB/17 (Item 17 from file: 155)
DIALOG(R)File 155:MEDLINE(R)

13042493 21629718 PMID: 11755786
Adult neural stem cells: plasticity and developmental potential.
Gritti Angela; Vescovi Angelo L; Galli Rossella

Institute for Stem Cell Research, DIBIT, S. Raffaele Hospital, Via Olgettina 58, Milan, Italy gritti.angela@hsr.it
Journal of physiology, Paris France Jan-Mar **2002**, 96 (1-2) p81-90, ISSN 0928-4257 Journal Code: 9309351
Document type: Journal Article; Review; Review, Tutorial
Languages: ENGLISH
Main Citation Owner: NLM
Record type: Completed
Stem cells play an essential role during the processes of embryonic **tissue** formation and development and in the maintenance of **tissue** integrity and renewal throughout adulthood. The differentiation potential of stem cells in adult tissues has been thought to be limited to cell lineages present in the organ from which they derive, but there is evidence that somatic stem cells may display a broader differentiation repertoire. This has been documented for **bone marrow** stem cells (which can give rise to muscle, hepatic and brain cells) and for muscle precursors, which can turn into **blood** cells. The adult central nervous system (CNS) has long been considered incapable of cell renewal and structural remodeling. Recent findings indicate that, even in postnatal and adult mammals, neurogenesis does occur in different brain regions and that these regions actually contain adult stem cells. These cells can be expanded both in vivo and ex vivo by exposure to different combinations of growth factors and subsequently give rise to a differentiated progeny comprising the major cell types of the CNS. Almost paradoxically, adult neural stem cells display a multipotency much broader than expected, since they can differentiate into non-CNS mesodermal-derivatives, such as **blood** cells and skeletal muscle cells. We **review** the recent findings documenting this unforeseen plasticity and unexpected developmental potential of somatic stem cells in general and of neural stem cells in particular. To better introduce these concepts, some basic notions on the functional properties of adult neural stem cells will also be discussed, particularly focusing on the emerging role of the microenvironment in determining and maintaining their peculiar characteristics.

9/3,AB/18 (Item 18 from file: 155)
DIALOG(R)File 155:MEDLINE(R)

12858885 21440405 PMID: 11556692
Human herpesvirus latency.
Cohrs R J; Gilden D H
Department of Neurology, University of Colorado, Health Sciences Center, Denver 80262, USA. randall.cohrs@uchsc.edu
Brain pathology (Zurich, Switzerland) (Switzerland) Oct **2001**, 11 (4) p465-74, ISSN 1015-6305 Journal Code: 9216781
Contract/Grant No.: AG06127; AG; NIA; NS32623; NS; NINDS
Document type: Journal Article; Review; Review, Tutorial
Languages: ENGLISH
Main Citation Owner: NLM
Record type: Completed
Herpesviruses are among the most successful human pathogens. In healthy individuals, primary infection is most often inapparent. After primary infection, the virus becomes latent in ganglia or **blood** mononuclear cells. Three major subfamilies of herpesviruses have been identified based on similar growth characteristics, genomic structure, and **tissue** predilection. Each herpesvirus has evolved its own unique ecological niche within the host that allows the maintenance of latency over the life of the individual (e.g. the adaptation to specific cell types in establishing latent infection and the mechanisms, including expression of different sets of genes, by which the virus remains latent). Neurotropic alphaherpesviruses become latent in dorsal root ganglia and reactivate to produce epidermal ulceration, either localized (herpes simplex types 1 and

2 or spread over several dermatomes (varicella-zoster virus). Human cytomegalovirus, the prototype betaherpesvirus, establishes latency in **bone marrow** -derived myeloid progenitor cells. Reactivation of latent virus is especially serious in transplant recipients and AIDS patients. Lymphotropic gammaherpesviruses (Epstein-Barr virus) reside latent in resting B cells and reactivate to produce various neurologic complications. This **review** highlights the alphaherpesvirus, specifically herpes simplex virus type 1 and varicella-zoster virus, and describes the characteristics of latent infection.

9/3,AB/19 (Item 19 from file: 155)
DIALOG(R)File 155:MEDLINE(R)

12637377 21589310 PMID: 11732696
Characterization of age-related changes in body weight and organ weights from birth to adolescence in humans.
Haddad S; Restieri C; Krishnan K
Groupe de Recherche en Toxicologie Humaine (TOXHUM), Faculte de Medecine, Universite de Montreal, Quebec, Canada.
Journal of toxicology and environmental health. Part A (England) Nov 23 **2001**, 64 (6) p453-64, ISSN 1528-7394 Journal Code: 100960995
Document type: Journal Article
Languages: ENGLISH
Main Citation Owner: NLM
Record type: Completed
The pharmacokinetics and **tissue** dose of chemicals may differ among individuals of a population, particularly between adults and children. The adult-children differences in pharmacokinetics arise from age-related changes in the physiological, biochemical, and physicochemical determinants of uptake and disposition of chemicals. The objectives of this study were to **review** the published literature to assemble data on the human body weight and organ weights as a function of age (specifically between birth and 18 yr old) and to analyze these data, in order to develop regression equations for calculating body weight and organ weights of children using age as the dependent function. The specific organs/tissues for which the data on age-related weight were obtained and analyzed include **blood**, adipose tissues, liver, lungs, brains, heart, kidneys, spleen, the reproductive organs (male: prostate gland, seminal vesicle, testes, and epididymis; female: ovaries, uterus, and uterine tubes), glands (adrenal, pituitary, thymus, pancreas, and thyroid), **bone marrow** (total and red), intestinal tract, stomach, muscle, skin (epidermis and dermis), and skeleton. In both male and female children, the sum of these organs is systematically lower than the body weight, and this discrepancy may be resolved with the additional availability and consideration of data on hypodermis weight. The equations and data on body weight and organ weights presented in this article should be useful for constructing age-specific, physiologically based pharmacokinetic models for children.

9/3,AB/20 (Item 20 from file: 155)
DIALOG(R)File 155:MEDLINE(R)

11375944 21489606 PMID: 11603050
[Stem cells in adults]
Stamceller hos voksne.
Borge O J; Funderud S
Avdeling for immunologi Det Norske Radiumhospital 0310 Oslo.
Tidsskrift for den Norske laegeforening (Norway) Aug 30 **2001**, 121 (20) p2398-401, ISSN 0029-2001 Journal Code: 0413423
Document type: Journal Article; Review; Review, Tutorial ; English Abstract

Languages: NORWEGIAN
Main Citation Owner: NLM
Record type: Completed

BACKGROUND: We present a literature **review** of the plasticity observed by adult stem cells. MATERIALS AND METHODS: We have reviewed the literature regarding stem cells from adults in order to summarise their ability to generate cells of other types than those of the **tissue** organ from which they were isolated. RESULTS: Adult stem cells have recently been demonstrated to terminally differentiate into cells of other tissues than those from which they were originally isolated. For example, **bone marrow** cells have been shown to generate liver, nerve, heart and skeletal muscle cells in addition to their well-known ability to produce **blood** and mesenchymal cells. INTERPRETATION: Most studies demonstrate a proof-of-principle in animal models; much more research is needed before adult stem cells can be utilised in human medicine. However, the published reports are encouraging and give reasons for a cautious optimism with regard to future clinical use.

9/3,AB/21 (Item 21 from file: 155)
DIALOG(R)File 155:MEDLINE(R)

11306755 21352649 PMID: 11459702
[Detection of micrometastases and circulating tumour cells using molecular biology technics in solid tumours]
Detection des micrometastases et des cellules tumorales circulantes par les techniques de biologie moleculaire dans les tumeurs solides.
Schleiermacher G; Delattre O
Inserm U.509, Pathologie moleculaire des cancers, Institut Curie, 26, rue d'Ulm, 75248 Paris Cedex 05.
Bulletin du cancer (France) Jun **2001**, 88 (6) p561-70, ISSN 0007-4551 Journal Code: 0072416
Document type: Journal Article ; English Abstract
Languages: FRENCH
Main Citation Owner: NLM
Record type: Completed

The extension of a cancer is a major prognostic factor which determines the therapeutic strategy. The occurrence of metastatic relapses in patients with initially localized tumours, despite a good local control, gives evidence for the possibility of spreading of occult tumour cells. The recent improvements of immunohistochemistry and molecular biology methods enable to detect tumour cells in various sites such as lymph nodes, **bone marrow** and **blood** with a considerably increased sensitivity as compared to conventional approaches. The markers used to detect tumour cells by PCR or RT-PCR can be either "**tissue**-specific" or "tumour specific". The drawback of the first group of markers is linked to the observation that **tissue**-specificity is frequently a relative concept leading to a high rate of false positives. Tumour-specific markers include gene fusions observed in various sarcomas, point mutations and presence of viral genomes in tumour cells. They are available and can be easily monitored in only a limited set of cancers. This **review** focuses on the molecular biology approaches which are used to detect occult tumour cells and on their clinical applications. The large number of studies which have been published in that field show that such a detection can be performed in a variety of target sites. However, results of studies performed on larger series of patients together with a better standardization of technics are necessary before they can be used for individual staging of patients.

9/3,AB/22 (Item 22 from file: 155)
DIALOG(R)File 155:MEDLINE(R)

11162710 21187373 PMID: 11292021
Elemental signals regulating eosinophil accumulation in the lung.
Foster P S; Mould A W; Yang M; Mackenzie J; Mattes J; Hogan S P; Mahalingam S; Mckenzie A N; Rothenberg M E; Young I G; Matthaei K I; Webb D C
Division of Biochemistry and Molecular Biology, John Curtin School of Medical Research, Australian National University, Acton, Canberra ACT. Paul.Foster@anu.edu.au
Immunological reviews (Denmark) Feb **2001**, 179 p173-81, ISSN 0105-2896 Journal Code: 7702118
Document type: Journal Article; Review; Review, Tutorial
Languages: ENGLISH
Main Citation Owner: NLM
Record type: Completed
In this **review** we identify the elemental signals that regulate eosinophil accumulation in the allergic lung. We show that there are two interwoven mechanisms for the accumulation of eosinophils in pulmonary tissues and that these mechanisms are linked to the development of airways hyperreactivity (AHR). Interleukin-(IL)-5 plays a critical role in the expansion of eosinophil pools in both the **bone marrow** and **blood** in response to allergen provocation of the airways. Secondly, IL-4 and IL-13 operate within the allergic lung to control the transmigration of eosinophils across the vascular bed into pulmonary tissues. This process exclusively promotes **tissue** accumulation of eosinophils. IL-13 and IL-4 probably act by activating eosinophil-specific adhesion pathways and by regulating the production of IL-5 and eotaxin in the lung compartment. IL-5 and eotaxin co-operate locally in pulmonary tissues to selectively and synergistically promote eosinophilia. Thus, IL-5 acts systemically to induce eosinophilia and within tissues to promote local chemotactic signals. Regulation of IL-5 and eotaxin levels within the lung by IL-4 and IL-13 allows Th2 cells to elegantly co-ordinate **tissue** and peripheral eosinophilia. Whilst the inhibition of either the IL-4/IL-13 or IL-5/eotaxin pathways resulted in the abolition of **tissue** eosinophils and AHR, only depletion of IL-5 and eotaxin concurrently results in marked attenuation of pulmonary inflammation. These data highlight the importance of targeting both IL-5 and CCR3 signalling systems for the resolution of inflammation and AHR associated with asthma.

9/3,AB/23 (Item 23 from file: 155)
DIALOG(R)File 155:MEDLINE(R)

11118746 21128116 PMID: 11222984
Molecular characterization of minimal residual cancer cells in patients with solid tumors.
Bockmann B; Grill H J; Giesing M
Institute for Molecular NanoTechnology, Berghauser Strasse 295, 45659, Recklinghausen, Germany.
Biomolecular engineering (Netherlands) Mar **2001**, 17 (3) p95-111, ISSN 1389-0344 Journal Code: 100928062
Document type: Journal Article; Review; Review, Tutorial
Languages: ENGLISH
Main Citation Owner: NLM
Record type: Completed
The failure to reduce the mortality of patients with solid tumors is mainly a result of the early dissemination of cancer cells to secondary sites, which is usually missed by conventional diagnostic procedures used for tumor staging. PCR was shown to be superior to conventional techniques in detecting circulating tumor cells and micrometastases allowing the identification of one tumor cell in up to 10(7) normal cells in various sources such as **blood**, **bone marrow**, lymph nodes, urine or stool. The methods used are based on the detection of either genomic

alterations in oncogenes and tumor suppressor genes or on the mRNA expression of **tissue** specific and tumor associated genes. The additional implementation of techniques for cancer cell purification had a significant impact on analytical sensitivity and specificity of MRCC detection. For patients with e.g. melanoma, breast, colorectal or prostate cancer it was demonstrated that the presence of disseminated cancer cells defines a subgroup of patients with reduced time to recurrence. The possibility to use easily accessible body fluids as a source for MRCC detection enables longitudinal observations of the disease. In this **review** we discuss the potential of molecular characterization of MRCC as a tool to improve prognostication, therapy selection and drug targeting as well as therapy monitoring.

9/3,AB/24 (Item 24 from file: 155)
DIALOG(R)File 155:MEDLINE(R)

11384106 21111345 PMID: 11173655
Regulatory pathways in **blood**-forming **tissue** with particular reference to gap junctional communication.
Rosendaal M; Krenacs T T
Department of Anatomy and Developmental Biology, University College London, Gower Street, London, WC1E 6BT, England. m.rosendaal@ucl.ac.uk
Pathology oncology research : POR (England) **2000**, 6 (4) p243-9
, ISSN 1219-4956 Journal Code: 9706087
Document type: Journal Article; Review; Review, Tutorial
Languages: ENGLISH
Main Citation Owner: NLM
Record type: Completed
Blood formation by pluripotent stem cells and their progeny is thought to be regulated by receptor-ligand interactions between cell-substrate, cell-cell and cell-matrix in the **bone marrow**. Primitive stem cells form progenitors and, in their turn, these give rise to haemopoietic progeny which are more specifically committed in that they can form progressively fewer types of **blood** cells. Recently we have established that direct cell-cell communication via gap junctions may be part of this regulatory system. Connexin43 gap junctions metabolically couple the three dimensional meshwork of **bone marrow** stromal cells to form a functional syncytium in which some **blood**-forming cells are also coupled. The expression of gap junctions in the **bone marrow** is markedly upregulated when there is an urgent and substantial demand for **blood** -formation; for example, following cytotoxic injury after 5-fluorouracil or irradiation; or during neonatal **blood** -formation and in the epiphysis of growing bones. Chemical blockade of gap junctions blocks **blood** -formation in long-term cultures but is reversible after the blockade has been relieved. This short **review** highlights briefly the known regulatory mechanisms of **blood** -formation with especial attention to gap junctional communication.

9/3,AB/25 (Item 25 from file: 155)
DIALOG(R)File 155:MEDLINE(R)

10978346 20535626 PMID: 11085176
Cell migration and the anatomic control of thymocyte precursor differentiation.
Prockop S; Petrie H T
Memorial Sloan-Kettering Cancer Center, New York 10021, USA. Prockop Susan/mskcc PEDS@mskmail.mskcc.org
Seminars in immunology (UNITED STATES) Oct **2000**, 12 (5) p435-44, ISSN 1044-5323 Journal Code: 9009458

Document type: Journal Article; Review; Review, Tutorial
Languages: ENGLISH
Main Citation Owner: NLM
Record type: Completed
The thymus performs several essential functions during the steady-state production of T lymphocytes in adults, including expansion of the precursor pool, differentiation into multiple lineages and screening for TCRs with restricted specificities. Other than those functions attributed to the TCR, most of the factors that control these processes remain undefined. One potential mechanism for such control may be related to the movement of precursor cells between distinct anatomical compartments in the thymus. Histological studies show that the majority of CD4- CD8- cells are found in the subcapsular region. However; vascular tissues that support the migration of precursor cells into the thymus (postcapillary venules) are located deep in the **tissue**, near the cortico-medullary junction. This implies that **blood** -borne cells entering the thymus must transit outward across the cortex in order to accumulate in the SCR. Differentiation of DN cells into the CD4+ 8+ stage correlates with a reversal in polarity and migration inward, while mature cells ultimately transit the CMJ in the opposite direction of cells first entering the organ. Here we **review** evidence for a model in which differentiation is induced and proliferation is controlled by this progressive translocation of immature precursors through discrete stromal compartments. In addition, we attempt to summarize what is known about the molecular mechanisms that may support polarized migration of early CD4- 8- thymocytes in the adult, as well as how and where the relevant differentiative and/or proliferative signals may be compartmentalized.

9/3,AB/26 (Item 26 from file: 155)
DIALOG(R)File 155:MEDLINE(R)

10973803 20513513 PMID: 11061435
Developmental control of chondrogenesis and osteogenesis.
Cancedda R; Castagnola P; Cancedda F D; Dozin B; Quarto R
Istituto Nazionale per la Ricerca sul Cancro, Centro di Biotecnologie Avanzate, Genova, Italy. cancedda@ermes.cba.unige.it
International journal of developmental biology (SPAIN) **2000**, 44 (6 Spec No) p707-14, ISSN 0214-6282 Journal Code: 8917470
Document type: Journal Article; Review; Review, Tutorial
Languages: ENGLISH
Main Citation Owner: NLM
Record type: Completed
During vertebrate embryogenesis, bones of the vertebral column, pelvis, and upper and lower limbs, are formed on an initial cartilaginous model. This process, called endochondral ossification, is characterized by a precise series of events such as aggregation and differentiation of mesenchymal cells, and proliferation, hypertrophy and death of chondrocytes. **Bone** formation initiates in the collar surrounding the hypertrophic cartilage core that is eventually invaded by **blood** vessels and replaced by **bone** **tissue** and **bone** **marrow**. Over the last years we have extensively investigated cellular and molecular events leading to cartilage and **bone** formation. This has been partially accomplished by using a cell culture model developed in our laboratory. In several cases observations have been confirmed or directly made in the developing embryonic **bone** of normal and genetically modified chick and mouse embryos. In this article we will **review** our work in this field.

9/3,AB/27 (Item 27 from file: 155)
DIALOG(R)File 155:MEDLINE(R)

10947023   20506697   PMID: 11055506

Bone marrow or peripheral **blood** as a source of stem cells for allogeneic transplants.

Bacigalupo A; Frassoni F; Van Lint M T

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Current opinion in hematology UNITED STATES Nov **2000**, 7 6 p343-7, ISSN 1065-6251 Journal Code: 9430802

Document type: Journal Article; Review; Review, Tutorial

Languages: ENGLISH

Main Citation Owner: NLM

Record type: Completed

Peripheral **blood** stem cell transplants are being increasingly used in the allogeneic setting and are often preferred to the conventional **bone marrow** source. The aim of this report is to **review** available data on peripheral **blood** versus **bone marrow** hematopoietic stem cell transplantation. The discussion is restricted to HLA-identical sibling transplants receiving unmanipulated grafts. This is because data with appropriate follow-up are available only for this type of comparison: we have preliminary data on the use of peripheral **blood** from unrelated donors, and on the use of T-cell depletion/CD34+ selection methods. The latter are evolving rapidly and it may be difficult to find a concurrent group of patients receiving T-cell depleted or CD34-selected **marrow** . The results of retrospective and prospective studies are similar: hematologic and immune recovery are faster after peripheral **blood** grafts, acute graft-versus-host disease is comparable, whereas chronic graft-versus-host disease is increased in recipients of peripheral **blood** transplants. Transplant-related mortality is similar in the two groups, whereas disease recurrence is lower after peripheral **blood** grafts. The general opinion is that peripheral **blood** grafts are indicated for patients with advanced disease, whereas for patients with early-phase disease the two sources may give comparable results.

9/3,AB/28   (Item 28 from file: 155)

DIALOG(R)File 155:MEDLINE(R)

10915748   20450912   PMID: 10980636

Cytokines and hemostasis.

Grignani G; Maiolo A

Department of Internal Medicine and Medical Therapy, Institute for Medical Care "Citta di Pavia", Viale Parco Vecchio, 27, 27100 Pavia, Italy. grignani@unipv.it

Haematologica (ITALY) Sep **2000**, 85 (9) p967-72, ISSN 0390-6078 Journal Code: 0417435

Document type: Journal Article; Review; Review, Tutorial

Languages: ENGLISH

Main Citation Owner: NLM

Record type: Completed

BACKGROUND AND OBJECTIVES: Cytokines are low molecular weight polypeptides that act as pleiotropic mediators of inflammation and may contribute significantly to regulation of hemostatic balance in both physiologic and pathologic conditions. The purpose of this **review** is to underline the most significant progresses recently achieved in this rapidly growing area. DESIGN AND METHODS: The authors have been involved both at home and abroad in experimental and clinical research in this field for years and have contributed original papers in peer-reviewed journals. In addition, the material examined in the present **review** includes articles published in journals covered by the Science Citation Index and Medline. RESULTS: **Tissue** factor, a transmembrane glycoprotein that serves as a surface receptor for coagulation factor VIIa, plays a key role in the initiation of coagulation processes. Very little, if any,

tissue factor activity is detectable in normal conditions on the cell surface of monocytes and endothelial cells. However, upon proper stimulation by a number of agents such activity may be expressed in these cells, which can then contribute significantly to clotting activation. Pro-inflammatory cytokines IL-1, IL-6 and TNF are effective inducers of **tissue** factor upregulation and may trigger endothelial cells to change their antithrombotic properties into a procoagulant, clot-promoting state. Indeed, much experimental and clinical evidence has been accumulated to suggest that cytokines play a key role in the pathophysiology of hemostatic abnormalities in different disease states. These include, inter alia, the coagulopathy observed during septicemia, the veno-occlusive disease of the liver after **bone marrow** transplantation, the prothrombotic state associated with atherosclerotic vessels, the occurrence of deep venous thrombosis after major abdominal surgery and the thrombotic tendency of patients with cancer. Several new antithrombotic strategies based on these new concepts have been attempted in experimental models of thrombosis and also in man. Examples of new possible antithrombotic agents are the **tissue** factor pathway inhibitor, Fab fragments of monoclonal antibodies directed against factor VII or factor VIIa, mutant forms of biologically inactive **tissue** factor and inhibition of cytokines involved in the regulation of **tissue** factor expression. Many of these studies have produced positive or interesting results, although more must be learned before the appropriate drug and the adequate dose are defined in the different clinical situations. CONCLUSIONS: Pro-inflammatory cytokines (IL-1, IL-6 and TNF) play a key role in **tissue** factor expression on monocytes and on endothelial cells and contribute significantly to regulation of hemostatic balance in physiologic and pathologic conditions. This effect is of great interest from both speculative and practical viewpoints.

9/3,AB/29 (Item 29 from file: 155)
DIALOG(R)File 155:MEDLINE(R)

10835064 20396679 PMID: 10936415
PPARgamma: observations in the hematopoietic system.
Greene M E; Pitts J; McCarville M A; Wang X S; Newport J A; Edelstein C; Lee F; Ghosh S; Chu S
Department of Cancer Biology/Pathology, Children's Memorial Institute for Education and Research (CMIER), Chicago, IL 60614, USA. mgreene@nwu.edu
Prostaglandins & other lipid mediators (UNITED STATES) Jun **2000**, 62 (1) p45-73, ISSN 1098-8823 Journal Code: 9808648
Contract/Grant No.: K08 DK02309; DK; NIDDK
Document type: Journal Article; Review; Review, Tutorial
Languages: ENGLISH
Main Citation Owner: NLM
Record type: Completed
Human Peroxisome Proliferator-Activated Receptor gamma (PPARgamma) was originally cloned from a human **bone marrow** library. What role does this ligand activated transcription factor play in hematopoiesis and the immune system? We note that: a) PPARgamma has potential to interact/interfere or synergize with retinoid biology, b) fatty acids and a prostaglandin have been identified as ligands, and c) lymphocytes, monocytes and neutrophils use fatty acids as a major source of energy production, d) PPARgamma has been shown to oppose TNFalpha and down regulate cytokine production in monocytes. Therefore, we undertook a **review** of the literature and an expression survey of PPARgamma in a number of major organs and cells involved in the hematopoietic system, for the purpose of building a database towards understanding the role and function of PPARgamma gene regulation in the developing **blood** and immune systems. PPARgamma is expressed before mesodermal induction in **tissue** in and around Spemann's organizer in the xenopus blastocyst, in erythroid precursors of **blood** islands and in the circulation of

the day 10.0 murine embryo, in human 19 week fetal liver, in some but not all murine and human **bone marrow** erythroid, myeloid, and monocytoid progenitors, **bone marrow** stromal cells and adipocytes, osteoblasts, endothelial cells, some T, and B lymphocytes, monocytes, macrophages, and other monocytic derivatives. It can be found in the cells of Peyer's patches, lymphoid follicles, spleen, and thymus. It is not clear if it is ever or transiently expressed in megakaryocytes, mast cells, or neutrophils. Based on the above data and a **review** of the literature, PPARgamma seems to play a role during the elicitation of immune responses. We propose PPARgamma may be involved in changes in energy states required during activation and development of many cell types involved, and has additional immunologically relevant effects in erythroid, myeloid, monocytic, T and B lymphocytic, stromal, and endothelial cell function.

9/3,AB/30 (Item 30 from file: 155)
DIALOG(R)File 155:MEDLINE(R)

10822088 20359891 PMID: 10899701
Pharmacology and physiology of melatonin in the reduction of oxidative stress in vivo.
Reiter R J; Tan D X; Qi W; Manchester L C; Karbownik M; Calvo J R
Department of Cellular and Structural Biology, The University of Texas Health Science Center, San Antonio, TX 78229-3900, USA. reiter@uthscsa.edu
Biological signals and receptors (SWITZERLAND) May-Aug **2000**, 9 (3-4) p160-71, ISSN 1422-4933 Journal Code: 9808792
Document type: Journal Article; Review; Review, Tutorial
Languages: ENGLISH
Main Citation Owner: NLM
Record type: Completed
This brief resume summarizes the evidence which shows that melatonin is a significant free radical scavenger and antioxidant at both physiological and pharmacological concentrations in vivo. Surgical removal of the pineal gland, a procedure which lowers endogenous melatonin levels in the **blood**, exaggerates molecular damage due to free radicals during an oxidative challenge. Likewise, providing supplemental melatonin during periods of massive free radical production greatly lowers the resulting **tissue** damage and dysfunction. In the current **review**, these findings are considered in terms of neurodegenerative diseases, cancer, ischemia/reperfusion injury and aging. Besides being a highly effective direct free radical scavenger and indirect antioxidant, melatonin has several features that make it of clinical interest. Thus, melatonin is readily absorbed when it is administered via any route, it crosses all morphophysiological barriers, e.g., **blood**-brain barrier and placenta, with ease, it seems to enter all parts of every cell where it prevents oxidative damage, it preserves mitochondrial function, and it has low toxicity. While **blood** melatonin levels are normally low, **tissue** levels of the indoleamine can be considerably higher and at some sites, e.g., in **bone marrow** cells and bile, melatonin concentrations exceed those in the **blood** by several orders of magnitude. What constitutes a physiological level of melatonin must be redefined in terms of the bodily fluid, **tissue** and subcellular compartment being examined. Copyright 2000 S. Karger AG, Basel

9/3,AB/31 (Item 31 from file: 155)
DIALOG(R)File 155:MEDLINE(R)

10756131 20306179 PMID: 10846824
Ex vivo expansion of megakaryocytic cells.
Maurer A M; Liu Y; Caen J P; Han Z C
National Laboratory of Experimental Hematology, Chinese Academy of

Medical Sciences, Tianjin, People's Republic of China.
International journal of hematology (IRELAND) Apr **2000**, 71 (3)
p203-10, ISSN 0925-5710 Journal Code: 9111627
Document type: Journal Article; Review; Review, Tutorial
Languages: ENGLISH
Main Citation Owner: NLM
Record type: Completed

The use of platelet transfusion to ensure the recovery of thrombopoiesis in patients constitutes high-cost support. The identification and cloning of recombinant human thrombopoietin (TPO) and the development of efficient methods of purification of hematopoietic stem cells and progenitor cells have ameliorated the development of strategies of ex vivo expansion of megakaryocyte (MK) progenitor cells and mature MKs. Synergistic combinations of cytokines including TPO, interleukin (IL)-1, IL-3, IL-11, stem cell factor, and FLT-3 ligand induce the ex vivo expansion of colony-forming unit-MK progenitors and MKs from cytokine-mobilized peripheral **blood** cells, **bone marrow**, and cord **blood** CD34+ cells. Depending on the various culture conditions, i.e., combinations of growth factors, initial concentration of CD34+, serum or serum-free cultures, and/or oxygen tensions, the expansion-fold of MKs and their progenitor cells vary greatly. The clinical applications of the reinfusion of ex vivo-generated MK cells have been investigated successfully in cancer patients following high-dose chemotherapy. This **review** reports the latest information concerning ex vivo expansion of MKs and the current status of clinical trials.

9/3,AB/32 (Item 32 from file: 155)
DIALOG(R)File 155:MEDLINE(R)

10702161 20246323 PMID: 10786651
Granulocyte colony-stimulating factor versus granulocyte-macrophage colony-stimulating factor for collection of peripheral **blood** progenitor cells from healthy donors.
Fischmeister G; Gadner H
St. Anna Children's Hospital and Children's Cancer Research Institute, Vienna, Austria.
Current opinion in hematology (UNITED STATES) May **2000**, 7 (3)
p158-5, ISSN 1065-6251 Journal Code: 9430802
Document type: Journal Article; Review; Review, Tutorial
Languages: ENGLISH
Main Citation Owner: NLM
Record type: Completed

The harvesting of peripheral **blood** progenitor cells (PBPCs) after granulocyte colony-stimulating factor or granulocyte-macrophage colony-stimulating factor stimulation instead of **bone marrow** in healthy donors has become increasingly popular. Donors, given the choice between **bone marrow** and PBPC donation, often prefer cytapheresis because of the easier access, no necessity for general anesthesia, and no multiple **bone marrow** punctures. In addition, accelerated engraftment and immunomodulation by granulocyte colony-stimulating factor-mobilized PBPCs are advantageous for the recipient. However, because of donor inconvenience and poor mobilization, there is a need to develop improved procedures. Aspects such as durability of hematopoietic engraftment, characterization of the earliest stem cell, and composition of PBPCs are not yet well defined, and international donor registration and follow-up must be considered when evaluating long-term safety profiles in healthy donors. This **review** concentrates on the most significant developments on mobilization of PBPCs published during the past year.

9/3,AB/33 (Item 1 from file: 5)

DIALOG P File 5:Biosis Previews R

14107414 BIOSIS NO.: 200300101443
II. Glutamine and glutamate.
AUTHOR: Tapiero H a ; Mathe G; Couvreur P; Tew K D
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JOURNAL: Biomedicine & Pharmacotherapy 56 9 :p446-457 November 2002
2002
MEDIUM: print
ISSN: 0753-3322
DOCUMENT TYPE: Literature Review
RECORD TYPE: Abstract
LANGUAGE: English

ABSTRACT: Glutamine and glutamate with proline, histidine, arginine and ornithine, comprise 25% of the dietary amino acid intake and constitute the "glutamate family" of amino acids, which are disposed of through conversion to glutamate. Although glutamine has been classified as a nonessential amino acid, in major trauma, major surgery, sepsis, **bone marrow** transplantation, intense chemotherapy and radiotherapy, when its consumption exceeds its synthesis, it becomes a conditionally essential amino acid. In mammals the physiological levels of glutamine is 650 mumol/l and it is one of the most important substrate for ammoniagenesis in the gut and in the kidney due to its important role in the regulation of acid-base homeostasis. In cells, glutamine is a key link between carbon metabolism of carbohydrates and proteins and plays an important role in the growth of fibroblasts, lymphocytes and enterocytes. It improves nitrogen balance and preserves the concentration of glutamine in skeletal muscle. Deamidation of glutamine via glutaminase produces glutamate a precursor of gamma-amino butyric acid, a neurotransmission inhibitor. L-Glutamic acid is a ubiquitous amino acid present in many foods either in free form or in peptides and proteins. Animal protein may contain from 11 to 22% and plants protein as much as 40% glutamate by weight. The sodium salt of glutamic acid is added to several foods to enhance flavor. L-Glutamate is the most abundant free amino acid in brain and it is the major excitatory neurotransmitter of the vertebrate central nervous system. Most free L-glutamic acid in brain is derived from local synthesis from L-glutamine and Kreb's cycle intermediates. It clearly plays an important role in neuronal differentiation, migration and survival in the developing brain via facilitated Ca++ transport. Glutamate also plays a critical role in synaptic maintenance and plasticity. It contributes to learning and memory through use-dependent changes in synaptic efficacy and plays a role in the formation and function of the cytoskeleton. Glutamine via glutamate is converted to alpha-ketoglutarate, an integral component of the citric acid cycle. It is a component of the antioxidant glutathione and of the polyglutamated folic acid. The cyclization of glutamate produces proline, an amino acid important for synthesis of collagen and connective **tissue**. Our aim here is to **review** on some amino acids with high functional priority such as glutamine and to define their effective activity in human health and pathologies.

2002

9/3,AB/34 (Item 2 from file: 5)
DIALOG(R)File 5:Biosis Previews R

14101556 BIOSIS NO.: 200300098585
The role of hepatocytes and oval cells in liver regeneration and

repopulation.

AUTHOR: Fausto Nelson a ; Campbell Jean S
AUTHOR ADDRESS: a Department of Pathology, University of Washington, Seattle, WA, USA**USA E-Mail: nfausto-u.washington.edu

JOURNAL: Mechanisms of Development 120 (1):p117-130 January 2003
2003
MEDIUM: print
ISSN: 0925-4773
DOCUMENT TYPE: Literature Review
RECORD TYPE: Abstract
LANGUAGE: English

ABSTRACT: The liver has the unique capacity to regulate its growth and mass. In rodents and humans, it grows rapidly after resection of more than 50% of its mass. This growth process, as well as that following acute chemical injury is known as liver regeneration, although growth takes place by compensatory hyperplasia rather than true regeneration. In addition to hepatocytes and non-parenchymal cells, the liver contains intrahepatic "stem" cells which can generate a transit compartment of precursors named oval cells. Liver regeneration after partial hepatectomy does not involve intra or extra-hepatic (hemopoietic) stem cells but depends on the proliferation of hepatocytes. Transplantation and repopulation experiments have demonstrated that hepatocytes, which are highly differentiated and long-lived cells, have a remarkable capacity for multiple rounds of replication. In this article, we **review** some aspects of the regulation of hepatocyte proliferation as well as the interrelationships between hepatocytes and oval cells in different liver growth processes. We conclude that in the liver, normally quiescent differentiated cells replicate rapidly after **tissue** resection, while intra-hepatic precursor cells (oval cells) proliferate and generate lineage only in situations in which hepatocyte proliferation is blocked or delayed. Although **bone marrow** stem cells can generate oval cells and hepatocytes, transdifferentiation is very rare and inefficient.

2003

9/3,AB/35 (Item 3 from file: 5)
DIALOG(R)File 5:Biosis Previews(R)

14066449 BIOSIS NO.: 200300060478
Aplastic anemia complicating systemic lupus erythematosus: Report of a case and **review** of the literature.

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JOURNAL: Rheumatology International 22 (6):p253-255 November 2002
2002
MEDIUM: print
ISSN: 0172-8172
DOCUMENT TYPE: Article
RECORD TYPE: Abstract
LANGUAGE: English

ABSTRACT: Aplastic anemia is a very unusual feature of systemic lupus erythematosus (SLE). A 32-year-old lady presented with generalized purpuric lesions and was diagnosed as having immune thrombocytopenic purpura. Fourteen months later, she developed progressive pancytopenia, arthritis of small joints, and oral ulcers. Investigations confirmed SLE with aplastic anemia. High-dose methylprednisolone therapy had been unsuccessful in controlling the pancytopenia. She had a progressive course and died due to septicemia. Even though pancytopenia is common in SLE, a **bone marrow** examination should be done in all cases of

persistent pancytopenia to exclude **bone marrow** aplasia. This will help in tailoring the treatment with more aggressive immunosuppressants.

2002

9/3,AB/36 (Item 4 from file: 5)
DIALOG(R)File 5:Biosis Previews(R)

14027723 BIOSIS NO.: 200300021752
Molecular and cellular mechanisms of donor cell-induced tolerance.
AUTHOR: George James F(a); Ahumada Leonik; Lu Ailing
AUTHOR ADDRESS: (a)University of Alabama at Birmingham, Birmingham, AL, 35294-0007, USA**USA E-Mail: jgeorge@uab.edu
JOURNAL: Immunologic Research 26 (1-3):p119-129 **2002**
MEDIUM: print
ISSN: 0257-277X
DOCUMENT TYPE: Literature Review
RECORD TYPE: Abstract
LANGUAGE: English

ABSTRACT: The induction of immunologic tolerance to solid organ allografts is a subject of intense investigation because of the morbidity and mortality associated with standard immunosuppressive therapy. One method that is currently in clinical and preclinical testing involves the transient ablation of recipient T cells using polyclonal antithymocyte sera or monoclonal anti-CD4/CD8 antibody treatment, followed by the posttransplant administration of donor **bone marrow** cells or of donor peripheral lymphoid populations. Recent studies in our laboratory have shown that the molecular and cellular basis of the prolongation of graft survival by donor cell administration depends on the cellular compartment from which the donor cells were derived. We provide here a brief **review** of these data followed by new data suggesting that the mode of peripheral and central selection is also dependent on the source from which the donor cells were derived.

2002

9/3,AB/37 (Item 5 from file: 5)
DIALOG(R)File 5:Biosis Previews(R)

14015324 BIOSIS NO.: 200300009353
Hodgkin's disease of the nasopharynx: Diagnostic and therapeutic approach with a **review** of the literature.
AUTHOR: Anselmo A P(a); Cavalieri E; Cardarelli L; Gianfelici V; Osti F M; Pescarmona E; Enrici R Maurizi
AUTHOR ADDRESS: (a)Dipartimento Biotecnologie Cellulari ed Ematologia, Universita "La Sapienza", Via Benevento, 6, 00161, Rome, Italy**Italy E-Mail: anselmo@bce.med.uniroma1.it
JOURNAL: Annals of Hematology 81 (9):p514-516 September 2002 **2002**
MEDIUM: print
ISSN: 0939-5555
DOCUMENT TYPE: Article
RECORD TYPE: Abstract
LANGUAGE: English

ABSTRACT: The lymphoid tissues of Waldeyer's ring, including the nasopharynx, are rarely involved in Hodgkin's disease (HD). Between March 1977 and July 2001, about 2150 patients affected by HD were observed in our institute; 7 of them (0.32%), all male patients, had HD of the

nasopharynx. They had no symptoms and **blood** tests were normal. All patients were treated with chemotherapy and or radiotherapy and achieved complete remission. At a median follow up of 72 months, they are alive and in continuous complete remission. We conclude that Hodgkin's disease of the nasopharynx is a rare and predominantly male disease with a particularly favorable prognosis. **Bone marrow** biopsy could be avoided. We believe that two to four cycles of a chemotherapeutic regimen and involved field radiotherapy at an intermediate-high dosage 25-30 Gy could be the first line treatment for these patients.

2002

9/3,AB/38 (Item 6 from file: 5)
DIALOG(R)File 5:Biosis Previews(R)

14002195 BIOSIS NO.: 200200631016
The use of in situ **bone marrow** stem cells for the treatment of various degenerative diseases.
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AUTHOR ADDRESS: (a)Research and Development, Desert Lake Technologies, P.O. Box 489, Klamath Falls, OR, 97601**USA E-Mail: cdrapeau@desertlake.com
JOURNAL: Medical Hypotheses 59 (4):p422-428 October, **2002**
MEDIUM: print
ISSN: 0306-9877
DOCUMENT TYPE: Literature Review
RECORD TYPE: Abstract
LANGUAGE: English

ABSTRACT: The potential for **tissue** repair and regeneration is encouraging in the light of novel research on the plasticity of adult stem cells. Intense research efforts over the last 3 years have provided solid evidence for the continuous generation of many types of **tissue** cells from adult stem cells as a normal part of our physiology throughout development and adult life in mammals, including humans. This opens new therapeutic avenues for many clinical problems and provides alternative opportunities at a time when much attention has been brought to the issue of using embryonic stem cells for research purposes and for the development of treatments for various diseases. Embryonic stem cells are pluripotent cells characterized by nearly unlimited self-renewal and differentiation capacity. However, evidence has accumulated over the past few years, indicating that adult **bone marrow** stem cells might have pluripotent properties similar to those of embryonic stem cells. Based on a **review** of the literature we propose the hypothesis that in situ mobilization of stem cells from the **bone marrow** and their migration to various tissues is a normal physiological process of regeneration and repair and that therapeutic benefits can be generated with less invasive regimens than the removal and re-injection of stem cells, through the stimulation of normal stem cell migration. We further propose that effort should be made to identify natural compounds characterized by their ability to augment this normal process of mobilization and re-colonization of **bone marrow** stem cells for the potential treatment of various degenerative diseases.

2002

9/3,AB/39 (Item 7 from file: 5)
DIALOG(R)File 5:Biosis Previews(R)

13944915 BIOSIS NO.: 200200573736

Structure and function of **bone marrow** hemopoiesis: Mechanisms of response to ionizing radiation exposure.

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JOURNAL: Cancer Biotherapy & Radiopharmaceuticals 17 4 :p405 426
2002
MEDIUM: print
ISSN: 1084-9785
DOCUMENT TYPE: Literature Review
RECORD TYPE: Abstract
LANGUAGE: English

ABSTRACT: It is the purpose of this presentation to **review** the unique structure and function of **bone marrow** anchored hematopoiesis in their significance for its response mechanisms to an exposure to ionizing radiation. The ultimate objective of **bone marrow** hematopoiesis is to maintain in the peripheral **blood** a constant level of the different **blood** cell types (erythrocytes, granulocytes, platelets, lymphocytes, etc.). All of them have their particular turnover kinetics (such as granulocytes 120X109/d, erythrocytes 200X109/d or thrombocytes 150X109/d), are semi-autonomous in their steady state regulatory mechanisms and dependent on a life-long supply of mature cells from a stem cell pool with unlimited replicative and pluripotent differentiative potential. The present knowledge of hematopoietic cellular renewal is the result of years of basic experimental and clinical studies using radionuclides in various metabolic forms including 59Fe, 32P (DF 32p), 51Cr, 131I, 60Co, 3H (3HTdR) and 14C (14CTdR). To understand the physiology but in particular the radiation-pathophysiology, it is essential to recognize in detail the infrastructure of the **bone marrow** as a distinct unit. Indispensable for a life-long cell production is the capsule of the **marrow**-the **bone** cortex-, the arterial supply of **blood** connected to the sinusoidal microvascular architecture with its sinusoids contorti and recti as well as the central (cell collecting) sinusoids. It is further of importance to recognize the significance of nerval regulation of **blood** flow, characterized by myelinated and unmyelinated nerve fibers. The type of unique lining cells of the sinusoids is the prerequisite for the cell traffic between the hemopoietic parenchyma and the **blood**. This in turn cannot be achieved without, an alternative opening and closing of the sinusoidal segments which-in turn-requires a rigid long capsule to assure an-in toto-constant volume of each **bone marrow** unit. If a **bone marrow** unit is exposed to ionizing radiation, a perturbance of the balance between cellular growth pressure and **blood** flow dynamics can be observed, resulting in a special type of **bone marrow** hemorrhage and an "excess cell loss" that may result in an non-thrombopenic exhaustion of the stem cell pool. Of great importance is the question as to the mechanisms that allow the **bone marrow** hemopoiesis to act as one cell renewal system although the **bone marrow** units are distributed throughout more than 100 **bone marrow** areas or units in the skeleton. The observation that "the **bone marrow**" acts and reacts as "one organ" is due to the regulatory mechanisms: the humeral factors (such as erythropoietins, granulopoietins, thrombopoietins etc.), the nerval factors (central nervous regulation) and cellular factors (continuous migration of stem cells through the **blood** to assure a sufficient stem cell pool size in each **bone marrow** "sub-unit"). It should be recalled that the **bone marrow** functions as a physiological chimera and becomes established by the hematogeneic seeding of stem cells to a mesenchymal matrix during embryogenesis. The repopulation of the **bone marrow** after partial body irradiation, after strongly inhomogeneous radiation exposure or after total body exposure with stem cell transplantation can well be considered

as a repetition of the embryogenesis of **bone marrow** hemopoiesis with the key element of stem cells migrating via the **blood** to stromal sites of the **marrow** prepared to accept stem cells to home and start their replication and differentiation if the micro-environmental quality permits. In summary, the radiation biology of **bone marrow** hemopoiesis requires a thorough understanding of the physiology and pathophysiology of structure, function and regulation not only of the process of cellular renewal but also of the intricate infrastructure.

2002

9/3,AB/40 (Item 8 from file: 5)
DIALOG(R)File 5:Biosis Previews(R)

13921299 BIOSIS NO.: 200200550120
Nocardiosis in cancer patients.
AUTHOR: Torres Harrys A; Reddy Bhavananda T; Raad Issam I; Tarrand Jeffrey; Bodey Gerald P; Hanna Hend A; Rolston Kenneth V I; Kontoyiannis Dimitrios P(a)
AUTHOR ADDRESS: (a)Department of Infectious Diseases, Infection Control and Employee Health, University of Texas M. D. Anderson Cancer Center, 1515 Holcombe Boulevard, Box 402, Houston, TX, 77030**USA E-Mail: dkontoyi@mdanderson.org
JOURNAL: Medicine (Baltimore) 81 (5):p388-397 September, **2002**
MEDIUM: print
ISSN: 0025-7974
DOCUMENT TYPE: Article
RECORD TYPE: Abstract
LANGUAGE: English

ABSTRACT: Nocardiosis (NOC) is an important cause of infection in immunocompromised patients. However, large series in patients with cancer have not been described. We **review** the records of patients with cancer and NOC who were evaluated at The University of Texas M. D. Anderson Cancer Center, Houston, Texas, between 1988 and 2001, and we describe the incidence, microbiologic and clinical characteristics, treatment, and outcome of NOC in this population. Forty-two patients with a total of 43 episodes of NOC were identified (incidence of 60 cases of NOC per 100,000 admissions). Twenty-seven patients (64%) had hematologic malignancies. In 13 patients, NOC complicated **bone marrow** transplantation. Neutropenia was observed in 4 (10%) of 40 episodes with information available, and lymphopenia in 20 (50%) of 40 episodes. Patients had received steroids for 25 episodes (58%) and had received chemotherapy for 10 episodes (23%) within 30 days before the onset of NOC. Nine episodes of breakthrough NOC were identified in 7 (23%) of the 40 patients with information available. Pulmonary NOC was seen in 30 (70%) of 43 cases; soft-**tissue** NOC in 7 (16%); central venous catheter-related nocardemia in 3 (7%); and disseminated NOC, central nervous system NOC, and a perinephric abscess each in 1 (2%). Twenty-three percent of patients with pulmonary NOC had an acute presentation. Nocardia asteroides complex was the most common causative species (77%). Therapy for NOC was mainly concurrent trimethoprim/sulfamethoxazole and either a tetracycline or a beta-lactam. The median duration of treatment was 113 days (range, 10-600 d). Nine (60%) of 15 patients with outcome data died from NOC. NOC, although infrequent, is an important cause of morbidity and mortality in patients with cancer. It has pleomorphic manifestations, and it can be seen as a breakthrough infection. The present study confirms that timely diagnosis, the site of NOC, the type of Nocardia, the presence of comorbidities, and cytomegalovirus coinfection influence the outcome of patients with cancer and NOC.

2002

9/3,AB/41 (Item 9 from file: 5)
DIALOG(R)File 5:Biosis Previews(R)

13868359 BIOSIS NO.: 200200497180
Generalized crystal-storing histiocytosis associated with monoclonal gammopathy: Molecular analysis of a disorder with rapid clinical course and **review** of the literature.
AUTHOR: Lebeau Annette(a); Zeindl-Eberhart Evelyn; Mueller Eva-Christina; Mueller-Hoecker Josef; Jungblut Peter Roman; Emmerich Bertold; Loehrs Udo
AUTHOR ADDRESS: (a)Pathologisches Institut der Ludwig-Maximilians-Universitaet, Thalkirchner Str. 36, 80337, Muenchen** Germany E-Mail: a.lebeau@lrz.uni-muenchen.de
JOURNAL: Blood 100 (5):p1817-1827 September 1, **2002**
MEDIUM: print
ISSN: 0006-4971
DOCUMENT TYPE: Article; Literature Review
RECORD TYPE: Abstract
LANGUAGE: English

ABSTRACT: Crystal-storing histiocytosis (CSH) is a rare event in disorders associated with monoclonal gammopathy. The intracellular crystal formation is almost always accompanied by the expression of kappa light chains. However, the exact mechanism for the storage has not been clarified until now. We report a case of generalized CSH in a 73-year-old man who presented with IgA kappa paraproteinemia and paraproteinuria. The initially observed CSH in the **bone marrow** biopsy was associated with the clinical and pathomorphologic features of a monoclonal gammopathy of undetermined significance. The progression of disease could not be affected by steroid therapy and the patient died of septic shock 7 months after detection of CSH. At the time of autopsy there was evidence for multiple myeloma and generalized CSH. Two-dimensional gel electrophoresis of liver **tissue** combined with immunoblotting revealed the massive storage of heavy chains of alpha type and light chains of kappa type, each in a monoclonal pattern. Analysis of the stored kappa light chain by nanoelectrospray-ionization mass spectrometry indicated that it belongs to the variable kappaI variability subgroup. We identified some unusual amino acid substitutions including Leu59, usually important for hydrophobic interactions within a protein, at a position where it has never been previously described in plasma cell disorders. In conclusion, we present the first case of CSH with molecular identification of the stored kappa subgroup and detection of unusual amino acid substitutions. Our results suggest that conformational alterations induced by amino acid exchanges represent a crucial pathogenic factor in CSH.

2002

9/3,AB/42 (Item 10 from file: 5)
DIALOG(R)File 5:Biosis Previews(R)

13865440 BIOSIS NO.: 200200494261
Central and peripheral nerve regeneration by transplantation of Schwann cells and transdifferentiated **bone marrow** stromal cells.
AUTHOR: Dezawa Mari(a)
AUTHOR ADDRESS: (a)Department of Anatomy, Yokohama City University School of Medicine, 3-9 Fukuura, Kanazawa-ku, Yokohama, 236-0004**Japan E-Mail: dezawa@med.yokohama-cu.ac.jp

JOURNAL: Anatomical Science International 77 (1):p12 25 March, **2002**
MEDIUM: print
DOCUMENT TYPE: Literature Review
RECORD TYPE: Abstract
LANGUAGE: English

ABSTRACT: In contrast to the peripheral nervous system (PNS), little structural and functional regeneration of the central nervous system (CNS) occurs spontaneously following injury in adult mammals. The inability of the CNS to regenerate is mainly attributed to its own inhibitorial environment such as glial scar formation and the myelin sheath of oligodendrocytes. Therefore, one of the strategies to promote axonal regeneration of the CNS is to experimentally modify the environment to be similar to that of the PNS. Schwann cells are the myelinating glial cells in the PNS, and are known to play a key role in Wallerian degeneration and subsequent regeneration. Central nervous system regeneration can be elicited by Schwann cell transplantation, which provides a suitable environment for regeneration. The underlying cellular mechanism of regeneration is based upon the cooperative interactions between axons and Schwann cells involving the production of neurotrophic factors and other related molecules. Furthermore, tight and gap junctional contact between the axon and Schwann cell also mediates the molecular interaction and linking. In this **review**, the role of the Schwann cell during the regeneration of the sciatic (representing the PNS) and optic (representing the CNS) nerves is explained. In addition, the possibility of optic nerve reconstruction by an artificial graft of Schwann cells is also described. Finally, the application of cells not of neuronal lineage, such as **bone marrow** stromal cells (MSCs), in nerve regeneration is proposed. **Marrow** stromal cells are known as multipotential stem cells that, under specific conditions, differentiate into several kinds of cells. The strategy to transdifferentiate MSCs into the cells with a Schwann cell phenotype and the induction of sciatic and optic nerve regeneration are described.

2002

9/3,AB/43 (Item 11 from file: 5)
DIALOG(R)File 5:Biosis Previews(R)

13855157 BIOSIS NO.: 200200483978
Spontaneous remission of low-grade B-cell non-Hodgkin's lymphoma following withdrawal of methotrexate in a patient with rheumatoid arthritis: Case report and **review** of the literature.
AUTHOR: Baird Richard D(a); van Zyl-Smit Richard Nellis; Dilke Timothy; Scott Sara E; Rassam Saad M B
AUTHOR ADDRESS: (a)Department of Haematology, Queen Mary's Hospital, Sidcup, Kent, DA14 6LR**UK E-Mail: r.baird@icr.ac.uk
JOURNAL: British Journal of Haematology 118 (2):p567-568 August, **2002**
MEDIUM: print
ISSN: 0007-1048
DOCUMENT TYPE: Article
RECORD TYPE: Abstract
LANGUAGE: English

ABSTRACT: A 69-year-old woman, who had suffered from deforming rheumatoid arthritis since the age of 40 years, had been treated with methotrexate for 3 years. She presented with a 7 week history of neck lymphadenopathy. Biopsy revealed low-grade marginal-zone B-cell non-Hodgkin's lymphoma. Computerized tomography and **bone marrow** biopsy confirmed stage IIIA disease. Spontaneous complete remission of the lymphoma was achieved 14 months after withdrawing immune suppression with

methotrexate.

2002

9/3,AB/44 (Item 12 from file: 5)
DIALOG(R)File 5:Biosis Previews(R)

13819951 BIOSIS NO.: 200200448772
Lung epithelial stem cells.
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JOURNAL: Journal of Pathology 197 (4):p527-535 July, **2002**
MEDIUM: print
ISSN: 0022-3417
DOCUMENT TYPE: Literature Review
RECORD TYPE: Abstract
LANGUAGE: English

ABSTRACT: This **review** concentrates on recent evidence about lung stem cell origins and plasticity. The range of potential cells which can repopulate the injured lung, classically the basal and mucous secretory cells of the trachea, the Clara cells of the bronchiole, and the type II pneumocyte of the alveolus, has been extended to include the mucus-gland duct cells of the trachea and bronchus. Some evidence suggests that there are variant Clara cells that lack cytochrome P-450 and so are spared toxic activation of xenobiotics, and may aid bronchiolar repopulation after injury, such as with naphthalene. There may even be involvement of the neuroepithelial bodies or cells in this, though the evidence is not yet conclusive. The search for a resident pulmonary multipotent cell for repopulating any lung epithelium has not yet been successful. The picture remains similar to earlier conclusions, in that the local stem or precursor cell is the most likely to contribute to local needs in times of **tissue** damage. There remains a major challenge for lung cancer treatment, where high-dose chemo- or radio-therapy may be hoped to promote the seeding and repair of lung parenchyma by circulating **bone marrow** stem cells, as seen in liver models. Patient survival rates do not yet suggest that this occurs to any great extent, but this remains to be shown formally. The effects of prior fibrosis and tumour necrosis are probably confounding factors in this lack of rescue.

2002

9/3,AB/45 (Item 13 from file: 5)
DIALOG(R)File 5:Biosis Previews(R)

13799479 BIOSIS NO.: 200200428300
Cyclosporin A-induced autoimmunity in the rat: Central versus peripheral tolerance.
AUTHOR: Damoiseaux J G M C(a)
AUTHOR ADDRESS: (a)Department of Immunology, University Maastricht, 6200 MD, P.O. Box 616, Maastricht**Netherlands E-Mail: jdam@limm.azm.nl
JOURNAL: International Journal of Immunopathology and Pharmacology 15 (2): p81-87 May-August, **2002**
MEDIUM: print
ISSN: 0394-6320
DOCUMENT TYPE: Literature Review
RECORD TYPE: Abstract
LANGUAGE: English

ABSTRACT: Cyclosporin A-induced autoimmunity CsA AI is a thymus dependent and T cell mediated autoimmune disease that is readily induced in rodents and also occurs in humans. Induction of CsA-AI requires total body X-irradiation, rescue with syngeneic or autologous **bone marrow**, and subsequent cyclosporin A CsA administration for about 4 weeks. Because the induction protocol involves **bone marrow** transplantation BMT , CsA-AI is also referred to as syngeneic or autoimmune graft-versus-host disease GVHD . The CsA-AI model is being studied for three reasons. Firstly, the animal model, and in particular the chronic phase of the disease, has been reported to have several macroscopic and histopathologic similarities with human scleroderma. Secondly, CsA-AI is clinically and experimentally examined for its graft-versus-tumour potential against lympho-hematopoietic malignancies as well as metastatic breast cancer. And thirdly, CsA-AI has been very informative in terms of T cell development and tolerance induction, including central as well as peripheral control of autoreactivity. In the present **review**, a summary of the characteristics of CsA-AI will be given. Next, the supposed mechanism of CsA for interference with central tolerance induction will be presented. Finally, the role of peripheral tolerance, and in particular dominant T cell tolerance as mediated by regulatory T cells, will be discussed in relation to induction of CsA-AI as well as to strain-related resistance to CsA-AI.

2002

9/3,AB/46 (Item 14 from file: 5)
DIALOG(R)File 5:Biosis Previews(R)

13733361 BIOSIS NO.: 200200361182
Toxoplasmosis in **bone marrow** transplantation: A report of two cases and systematic **review** of the literature.
AUTHOR: Mele A; Paterson P J; Prentice H G; Leoni P; Kibbler C C(a)
AUTHOR ADDRESS: (a)Department of Microbiology, Medical School, Royal Free and University College, Rowland Hill Street, Royal Free Campus, London, NW3 2PF**UK
JOURNAL: Bone Marrow Transplantation 29 (8):p691-698 April 2, **2002**
MEDIUM: print
ISSN: 0268-3369
DOCUMENT TYPE: Article; Literature Review
RECORD TYPE: Abstract
LANGUAGE: English

ABSTRACT: Toxoplasma infection represents a rare but often fatal complication in **bone marrow** transplant (BMT) recipients. We report two cases of toxoplasmosis: one of successfully treated cerebral toxoplasmosis after peripheral **blood** stem cell transplantation, and a fatal case of pulmonary toxoplasmosis in a BMT recipient. We have systematically reviewed the 110 published cases of toxoplasmosis following BMT. We analyzed the pretransplant and clinical features of BMT recipients developing toxoplasmosis, together with the diagnostic procedures used and treatment given. By univariate and multivariate statistical analysis we analyzed the risk factors for diagnosis (during life vs post-mortem) and Toxoplasma-related mortality. Ante-mortem diagnosis was made in 47% of cases. Site of infection (P=0.02; odds ratio 10.9), presence of symptoms at onset (P=0.01) and conditioning regimen (P=0.04) were factors influencing whether the diagnosis was made before or after death. Overall mortality rate was 80% and that attributed to toxoplasmosis was 66%. Variables influencing outcome were: site of infection (P=0.02; odds ratio 5.28), day of onset (P=0.04) and conditioning regimen (P=0.04). Underlying disease (P=0.02; odds ratio 9.45), among patients diagnosed before death, was the most significant factor influencing outcome.

2002

9/3,AB/47 (Item 15 from file: 5)
DIALOG(R)File 5:Biosis Previews(R)

13721352 BIOSIS NO.: 200200350173
Human herpes virus-8 implicated in pathogenesis of multiple myeloma.
AUTHOR: Awedan Abdulnaser(a); Ramadan Ahlam
AUTHOR ADDRESS: (a)Klinika Hematologii, Onkologii i Chorob Wewnetrznych, ul. Banacha 1a, 02-097, Warszawa**Poland
JOURNAL: Acta Haematologica Polonica 33 (1):p67-73 **2002**
MEDIUM: print
ISSN: 0001-5814
DOCUMENT TYPE: Article
RECORD TYPE: Abstract
LANGUAGE: English

ABSTRACT: Human herpes virus-8 (HHV-8) is a gamma-herpes virus that was first identified in the Kaposi's sarcoma **tissue** of acquired immune deficiency syndrome patients. It has been implicated in the pathogenesis of Kaposi's sarcoma (KS), body cavity based lymphoma (BCBL) and multicentric Castleman's disease. HHV-8 genomic sequences were recently detected by polymerase chain reaction (PCR) and in situ hybridization in **bone marrow** stromal cells grown from multiple myeloma patients. The virus is also detectable in the peripheral **blood** enriched with dendritic cells of most multiple myeloma patients. HHV-8 is not detected in the peripheral **blood** of family members and sexual partners of myeloma patients. In addition, HHV-8 encodes a viral homologue of IL-6, which like its human counterpart is capable of stimulating growth and preventing apoptosis of murine and human myeloma cell lines, suggesting a role for HHV-8 in the pathogenesis of myeloma. However, the association between multiple myeloma and HHV-8 still remains controversial issue, because some investigators have been unable to find HHV-8 virus in **bone marrow** and peripheral **blood** of multiple myeloma patients. Moreover, serological assays have failed to detect HHV-8 expression in-patients with multiple myeloma and in sexual partners. Although some studies suggest a potential role of HHV-8 in pathogenesis of multiple myeloma, further work is required to establish this connection clearly. The establishment of such link could lead to major changes in how clinicians approach this fatal malignancy. The aim of this **review** is to summarize current knowledge about the role of HHV-8 in the pathogenesis of multiple myeloma.

2002

9/3,AB/48 (Item 16 from file: 5)
DIALOG(R)File 5:Biosis Previews(R)

13705489 BIOSIS NO.: 200200334310
Recent advances in and therapeutic potential of muscle-derived stem cells.
AUTHOR: O'Brien Kristine; Muskiewicz Kristina; Gussoni Emanuela(a)
AUTHOR ADDRESS: (a)Division of Genetics, Children's Hospital, 320 Longwood Ave, Boston, MA, 02115**USA E-Mail: gussoni@enders.tch.harvard.edu
JOURNAL: Journal of Cellular Biochemistry Supplement (38):p80-87 **2002**
MEDIUM: print
ISSN: 0733-1959
DOCUMENT TYPE: Literature Review
RECORD TYPE: Abstract
LANGUAGE: English

ABSTRACT: Over the past few years, issues related to the commitment and potential of reservoir precursor cells that reside in most tissues have been revisited. Many reports have documented either plasticity or de-differentiation of a number of precursor cells isolated from several tissues, including **bone marrow**, brain, and skeletal muscle. These findings have challenged the dogma that mononuclear cells derived from adult, post-mitotic tissues can differentiate and contribute only to the **tissue** from which they originate. Thus, much current research in stem cells is testing the therapeutic potential of these cells to deliver normal genes and their encoded proteins into damaged or injured tissues. This **review** will focus on muscle-derived precursor cells and their apparently heterogeneous nature and summarize some of the most recent findings and hypotheses on their characterization and practical use.

2002

9/3,AB/49 (Item 17 from file: 5)
DIALOG(R)File 5:Biosis Previews(R)

13603677 BIOSIS NO.: 200200232698
Multiple myeloma as the first manifestation of acquired immunodeficiency syndrome: A case report and **review** of the literature.
AUTHOR: Pouli A(a); Lemessiou H; Rontogianni D; Papanastassiou C; Tsakanikas S; Gerassimou A; Stamatelou M
AUTHOR ADDRESS: (a)10, Tirinthou Str, A., Glyfada, Athens, 165-61**Greece
E-Mail: apouli@altavista.com
JOURNAL: Annals of Hematology 80 (9):p557-560 September, **2001**
MEDIUM: print
ISSN: 0939-5555
DOCUMENT TYPE: Article
RECORD TYPE: Abstract
LANGUAGE: English

ABSTRACT: Various hematologic malignancies and solid tumors are increasingly diagnosed in patients with human immunodeficiency virus (HIV) infection and may be the presenting manifestation of acquired immunodeficiency syndrome (AIDS). Multiple myeloma, however, has never been reported as the presenting manifestation of AIDS. We report on a 34-year-old man who presented with back pain, paresthesias, paraparesis, vertebral bony disease, and an associated soft **tissue** mass. Biopsy of the mass revealed immature plasmacytes with very faint cytoplasmic expression of kappa light chains. **Bone marrow** biopsy revealed 25% infiltration with poorly characterized malignant cells and 15% polyclonal plasma cells. Immunofixation of serum and urine was positive for IgG kappa and kappa light chains, respectively. A **bone** survey revealed lesions in the skull, left femur **bone**, and the pelvis. The diagnosis of an anaplastic myeloma was made. Because of the poorly characterized nature of the malignant cells and the difficulties in immunophenotyping, serologic evaluation for HIV was undertaken and was positive. The concept of myeloma as an opportunistic neoplasm defining AIDS was considered. We discuss this view and recommend that patients with multiple myeloma with poorly characterized myeloma cells as well as difficulties in immunophenotyping should undergo testing for HIV infection.

2001

9/3,AB/50 (Item 18 from file: 5)
DIALOG(R)File 5:Biosis Previews(R)

13579239 BIOSIS NO.: 200200208060
Immunobiology of liver dendritic cells.
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PA, 15213**USA E-Mail: thomsonaw@msx.upmc.edu
JOURNAL: Immunology and Cell Biology 80 (1):p65-73 February, **2002**
MEDIUM: print
ISSN: 0818-9641
DOCUMENT TYPE: Literature Review
RECORD TYPE: Abstract
LANGUAGE: English

ABSTRACT: Dendritic cells (DC) are rare, **bone marrow**-derived antigen-presenting cells that play a critical role in the induction and regulation of immune reactivity. In this article, we **review** the identification and characterization of liver DC, their ontogenic development, in vivo mobilization and population dynamics. In addition, we discuss the functions of DC isolated from liver **tissue** or celiac lymph, or propagated in vitro from liver-resident haemopoietic stem/progenitor cells. Evidence concerning the role of DC in viral hepatitis, liver tumours, autoimmune liver diseases, granulomatous inflammation and the outcome of liver transplantation is also discussed.

2002

9/3,AB/51 (Item 19 from file: 5)
DIALOG(R)File 5:Biosis Previews(R)

13578319 BIOSIS NO.: 200200207140
Role of fine-needle aspirates of focal lung lesions in patients with hematologic malignancies.
AUTHOR: Wong Philip W; Stefanec Tihomer; Brown Karen; White Dorothy A(a)
AUTHOR ADDRESS: (a)Pulmonary Medicine, Memorial Sloan-Kettering Cancer
Center, 1275 York Ave, C-678, New York, NY, 10021**USA E-Mail:
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JOURNAL: Chest 121 (2):p527-532 February, **2002**
MEDIUM: print
ISSN: 0012-3692
DOCUMENT TYPE: Article
RECORD TYPE: Abstract
LANGUAGE: English

ABSTRACT: Objectives: To evaluate the yield and safety of transthoracic fine-needle aspiration (FNA) in the diagnosis of pulmonary disease in patients with hematologic malignancy. Design: Retrospective chart **review**. Setting: Tertiary-care medical center. Patients: Sixty-seven patients with a hematologic malignancy or after **bone marrow** transplantation (BMT) for a hematologic malignancy who underwent a total of 71 FNAs for diagnosis of an unexplained parenchymal lung lesion from January 1, 1991, to June 30, 1999. Results: The underlying malignancy was lymphoma in 42 patients (63%), leukemia in 8 patients (12%), after allogeneic BMT in 12 patients (18%), after autologous BMT in 3 patients (4%), and other diseases in 2 patients. Radiographs showed focal abnormalities in all cases, and were nodules in 37%, masses in 37%, focal infiltrates in 21%, and cavitary lesions in 5%. The yield of FNA for a finding specific infection or cancer was 56% (40 of 71 FNAs). The FNA with inflammatory changes was clinically sufficient in another 11 patients for a total yield of 72% (51 of 71 FNAs). The yield for lung cancer was 90% (9 of 10 FNAs), for pulmonary lymphoma was

68% (21 of 31 FNAs), and for infection was 67% (10 of 15 FNAs). Complications occurred in 18 of 71 FNAs (25%), with pneumothorax in 14 patients (20%) and chest tube placement required in 4 patients (6%). Bleeding occurred in six patients (8%), including one death in a patient with abnormal hematologic parameters. Conclusion: Transthoracic FNA in patients with hematologic malignancy and focal lung lesions has an excellent yield for detecting cancer and a yield comparable to bronchoscopy for the diagnosis of infections. It should be considered a useful diagnostic tool in this setting.

2002

9/3,AB/52 (Item 20 from file: 5)
DIALOG(R)File 5:Biosis Previews(R)

13576938 BIOSIS NO.: 200200205809
Choosing an optimal radioimmunotherapy dose for clinical response.
AUTHOR: DeNardo Sally J(a); Williams Lawrence E; Leigh Bryan R; Wahl Richard L
AUTHOR ADDRESS: (a)Molecular Cancer Institute, University of California, Davis Medical Center, 1508 Alhambra Boulevard, Room 3100, Sacramento, CA, 95816**USA E-Mail: sjdenardo@ucdavis.edu
JOURNAL: Cancer 94 (4 Supplement):p1275-1286 February 15, **2002**
MEDIUM: print
ISSN: 0008-543X
DOCUMENT TYPE: Literature Review
RECORD TYPE: Abstract
LANGUAGE: English

ABSTRACT: Clinical trials have documented the single-agent efficacy of radioimmunotherapy (RIT) in lymphoma, and several combination therapy studies are now in progress. RIT agents are currently becoming generally available for clinical use in lymphoma therapy. Solid tumors, which are notoriously less responsive to any single agent, have demonstrated clinically useful responses, albeit temporary, and multimodality studies have been instituted. However, a sincere debate continues regarding the basic parameters to be used to define appropriate therapeutic dosing when using this modality in clinical cancer care. It is a good time to reevaluate relevant dose response information from preclinical and clinical RIT. Preclinical studies have demonstrated abundant evidence of dose response in tumor and normal **tissue** in homogenous model systems; however, substantive variation occurs between the dose responses of tumors with low and variable (or shed) antigen expression, as well as between histologically different tumor models. Clinical studies of various heavily pretreated patient populations given several very different RIT pharmaceuticals have led to disparate conclusions regarding patient dosing methods and dosimetric predictions of toxicity and efficacy. Single-study data on previously untreated lymphoma patients with similar histology has demonstrated a correlation of imaging dosimetry with toxicity and tumor response. High-dose therapy with **bone marrow** support has also demonstrated a high tumor response rate and nonmarrow normal organ toxicities that correlate with the calculated dose to those organs from imaging. In iodine-131 (131I)-anti-CD20 studies, 131I was demonstrated to have variable excretion, and estimated total-body radiation dose from tracer study proved a predictive surrogate for **marrow** toxicity. Yttrium-90 (90Y)-anti-CD20, which has little 90Y excretion from the body, demonstrated the injected dose per body weight to be more predictive of **marrow** toxicity than indium-111 (111In) tracer dosimetry methods in heavily pretreated patients, and showed maximal safety with standard mCi/kg therapy dosing. Variations in clinical RIT choices, dosing methods, and dosimetry methods emphasize the need to **review** the

relevant information to date. Future clinical trial designs, the sophistication of dosimetry, treatment planning, and clinical treatment decisions should all be focused on achieving the best benefit risk relationship for each patient.

2002

9/3,AB/53 (Item 21 from file: 5)
DIALOG(R)File 5:Biosis Previews(R)

13576053 BIOSIS NO.: 200200204874
Mucopolysaccharidoses and spinal cord compression: Case report and **review** of the literature with implications of **bone marrow** transplantation.
AUTHOR: Kachur Edward; Del Maestro Rolando(a)
AUTHOR ADDRESS: (a)London Health Sciences, 800 Commissioners Road East, RM 6109, Westminister Campus, London, ON, N6A 4G5**Canada E-Mail: delmaesr@lhsc.on.ca
JOURNAL: Neurosurgery (Baltimore) 47 (1):p223-229 July, **2000**
MEDIUM: print
ISSN: 0148-396X
DOCUMENT TYPE: Article; Literature Review
RECORD TYPE: Abstract
LANGUAGE: English

ABSTRACT: OBJECTIVE AND IMPORTANCE: We present a patient with mucopolysaccharidosis with spinal cord compression, and we **review** previously published cases. This is the first published case of a patient with mucopolysaccharidosis with spinal cord compression who has undergone **bone marrow** transplantation. CLINICAL PRESENTATION: A 2-year-old patient with Hurler syndrome underwent **bone marrow** transplantation. Although the **bone marrow** transplantation improved many of the systemic effects of Hurler syndrome, the patient presented at 8 years of age with a cervical myelopathy. Magnetic resonance imaging revealed soft **tissue** compression of the upper cervical cord. The literature **review** demonstrates that spastic tetraparesis, secondary to cervical cord compression, is the most common presentation of this subgroup of patients. INTERVENTION: A suboccipital craniectomy and C1-C5 laminectomy and decompression with duraplasty were performed. Pathological examination of compressive soft **tissue** and lamina was consistent with mucopolysaccharidosis. Postoperatively, the patient showed substantial improvement in neurological function. CONCLUSION: Mucopolysaccharidoses can induce a compressive "metabolic myelopathy." Decompressive procedures have shown significant improvement in neurological function in the majority of patients without spinal instability. **Bone marrow** transplantation may allow more patients with mucopolysaccharidoses to survive long enough to require neurosurgical treatment in the future. The effect of **bone marrow** transplantation on the prevention of spinal cord compression is unclear.

2000

9/3,AB/54 (Item 22 from file: 5)
DIALOG(R)File 5:Biosis Previews(R)

13523821 BIOSIS NO.: 200200152642
Hypogammaglobulinemia presenting as a non-secretory light chain myeloma.
AUTHOR: Cusano Gregory P a ; Rappaport Edward S a ; Ross William a ; Mott Frank

AUTHOR ADDRESS: a Pathology, Scott and White Memorial Hospital, Temple, TX **USA
JOURNAL: Blood 98 (11 Part 2):p296b November 16, **2001**
MEDIUM: print
CONFERENCE/MEETING: 43rd Annual Meeting of the American Society of Hematology, Part 2 Orlando, Florida, USA December 07-11, 2001
ISSN: 0006-4971
RECORD TYPE: Abstract
LANGUAGE: English

ABSTRACT: We present a 74 year old gentleman who presented with rib and sternal pain which was originally diagnosed as costochondritis. The treatment for this diagnosis was successful for a short period of time, but the pain returned. Radiologic studies demonstrated bilateral rib fractures. The pain worsened and further work-up including an MRI of the thoracolumbar spine revealed lytic lesions in T10 through T12 and compression fractures of T5 and T7. A soft **tissue** mass was also noted extending from T7 in the ventral epidural space causing posterior and right lateral displacement of the spinal cord. A CT guided biopsy af this mass demonstrated an increased number of plasma cells, but was not diagnostic of myeloma. During this same time period repeated serum protein electrophoretic and serum immunofixation studies were performed showig persistance of hypogammaglobulinemia without a paraprotein spike. Concentrated urine electrophoresis and urine immunofixtion were consistently negative for light chains. Eventually a **bone marrow** aspirate and biopsy were were done revealing the presence of massive infiltrations by small uniform plasma cells. The plasma cells replaced over 80% of the **marrow**. Immunohistochemical stain for kappa and lambda light chains showed an almost exclusive population of lamda positive plasma cells. This finding led to a diagnosis of non-secretory lambda light chain disease. This rare entity is present in only 1-4% of patients with myeloma. A **review** of the literature will be presented.

2001

9/3,AB/55 (Item 23 from file: 5)
DIALOG(R)File 5:Biosis Previews(R)

13523559 BIOSIS NO.: 200200152380
Increased microvessel density in non-Hodgkin's lymphoma (NHL) is localized to the site of disease changes in accordance with disease response.
AUTHOR: Wadleigh Martha(a); Niedzwiecki Donna(a); Davis Patty(a); Payne Nancy(a); Sen Filiz(a); Mann Karen P; Proia Alan; Hollis Donna(a); Rizzieri David A(a)
AUTHOR ADDRESS: (a)Division of Oncology and Stem Cell Transplantation, Duke University Medical Center, Durham, NC**USA
JOURNAL: Blood 98 (11 Part 2):p236b November 16, **2001**
MEDIUM: print
CONFERENCE/MEETING: 43rd Annual Meeting of the American Society of Hematology, Part 2 Orlando, Florida, USA December 07-11, 2001
ISSN: 0006-4971
RECORD TYPE: Abstract
LANGUAGE: English

ABSTRACT: Angiogenesis has been shown to correlate with metastatic potential in some neoplasms. Little is known, however, concerning changes in microvessel density (MVD) within different visceral tissues of the same patient, at the same point in time. Is angiogenesis increased only in the site of malignancy or in all **tissue** sites of the body? Furthermore, evaluation of changes over time in the same **tissue** sites, within the same patients has also not been carried out in depth.

Once angiogenesis is stimulated, does it always remain activated even when the disease is eradicated at that site? We reviewed 408 biopsy specimens from 100 patients and provide evidence for the role of increased angiogenesis in non-Hodgkin's lymphomas from multiple samples obtained over the courses of treatment within individual patients. Microvessels were detected using FVIII staining and t tests (student's or paired) were used for all comparisons below. Our work revealed that the baseline MVD of **marrow** is low (8.15 vessels/sq um, standard error .86; n=76 samples) and is similar to that published for normal lymph nodes. Lymph nodes involved with lymphoma have a sightly higher MVD (67.9 microvessels/sq um, SD 23.89; n=50) than **marrow** involved with lymphoma (49.6 microvessels/sq um, SD 17.35; n=38), though both are quite significantly elevated from the normal baseline MVD as shown above. These data allow us to compare involved and uninvolved sites over time and assess the changes in MVD as they relate to changes in disease status. We reviewed 24 paired biopsy samples taken from the same patient at the same time, with a different result (i.e. one site involved with lymphoma and the other site not involved). The mean MVD for this subgroup of involved sites was 74.6, SD 29.1, and was significantly higher than the uninvolved sites tested at the same time in the same patients (mean MVD 5.9 microvessels/sq um, SD 5.5, p<.0001). We reviewed 39 pairs of biopsy specimens in which the earlier specimen was involved with disease and the later one, post therapy, was not. There is a significant decrease in mean MVD from the initial involved biopsy compared to the later uninvolved biopsy (mean difference 52.1 microvessels/sq um, SD 30.3, p<.0001). Further, **review** of 22 paired samples reveled that in patients who had biopsies with no involvement initially and later developed involvement at that site had a significant increase in the MVD, corresponding to progressive disease (mean increase 50.76 microvessels/sq um, SD 25.79, p<.0001). These data reveal the dynamic nature of angiogenesis within the same patient, noting that the increase in angiogenesis in lymphoma appears localized to the site of disease. Further, the degree of angiogenesis continues to vary within patients, over time, in relation to the disease status of the site biopsied. This may have important implications as we target disease therapy with anti-angiogenesis agents. The unique properties of the stromal **tissue** within the disease site and how the stroma varies over time, rather than known circulating stimulants of angiogenesis, may be important to explore regarding the reasons the increased angiogenesis remains localized to the disease site rather than increased throughout the whole organism.

2001

9/3,AB/56 (Item 24 from file: 5)
DIALOG(R)File 5:Biosis Previews(R)

13522750 BIOSIS NO.: 200200151571
Plasmaphresis improves survival in drug induced thrombotic thrombocytopenic purpura: Systematic **review** of published case reports.
AUTHOR: Majhail Navneet S(a); Lichtin Alan E
AUTHOR ADDRESS: (a)Internal Medicine, Cleveland Clinic Foundation, Cleveland, OH**USA
JOURNAL: Blood 98 (11 Part 2):p60b-61b November 16, **2001**
MEDIUM: print
CONFERENCE/MEETING: 43rd Annual Meeting of the American Society of Hematology, Part 2 Orlando, Florida, USA December 07-11, 2001
ISSN: 0006-4971
RECORD TYPE: Abstract
LANGUAGE: English

ABSTRACT: Introduction: Thrombotic thrombocytopenic purpura (TTP) is a syndrome characterized by fever, neurologic symptoms, impaired renal

function, microangiopathic hemolytic anemia (MAHA) and thrombocytopenia. Drugs have been implicated in the etiology of TTP. We performed a systematic **review** of published case reports of drug induced TTP to characterize the symptomatology and to identify factors influencing overall survival. Materials and Methods: Articles were retrieved from the MEDLINE database (from 1966 through 31 July 2001) using the MeSH term thrombotic thrombocytopenic purpura with the attached subheading chemically induced (including drug induced as a keyword) and limits set for human only and English language only. Bibliographies of retrieved articles were crosschecked to identify additional reports. Criteria for excluding articles from further **review** were; (1) insufficient clinical data to establish the diagnosis of TTP (absence of thrombocytopenia or MAHA), (2) other possible causes of TTP could not be excluded (including presence of malignancy not in remission and history of **bone marrow** or solid organ transplant in the past), and (3) **review** articles and letters containing no patient case reports. Results: Total of 116 eligible patient case reports were retrieved from 32 articles. Ticlopidine (80%) was the most common drug associated with drug induced TTP followed by clopidogrel (8%). The median age at presentation was 64 years (range 15 to 90 years). TTP developed within a median of 21 days (range 1 to 330 days) of initiation of the offending agent. Common clinical manifestations on presentation included renal failure (79%), neuropsychiatric symptoms (72%), fever (57%), purpura (14%) and chest pain (6%). Only 10 (8.6%) patients presented with the classic pentad of TTP. The median Rose Eldor score observed was 5. There were 29 reported deaths from drug induced TTP. The use of plasmaphresis significantly improved overall survival in patients with drug induced TTP ($p<0.0001$). Age ($p=0.1584$), sex ($p=0.1072$) and Rose Eldor score ($p=0.4626$) were not observed to be significantly associated with increased mortality from drug induced TTP. Addition of corticosteroids to plasmaphresis did not improve survival ($p=0.0983$). Conclusions: Ticlopidine is the most common pharmacological agent associated with drug induced TTP. The classic pentad of fever, neurological symptoms, renal failure, thrombocytopenia and MAHA is not commonly seen in patients with drug induced TTP. Prompt recognition of the syndrome with initiation of plasmaphresis improves survival.

2001

9/3,AB/57 (Item 25 from file: 5)
DIALOG(R)File 5:Biosis Previews(R)

13522749 BIOSIS NO.: 200200151570
Acute, severe thrombocytopenia and sarcoidosis.
AUTHOR: Li Zujun(a); Halperin I(a); Astrow Alan(a); Kempin Sanford(a); Zhang Yuanchao; McBrien J(a); Tsai C(a)
AUTHOR ADDRESS: (a)Department of Medicine, Saint Vincents Hospital and Medical Center, New York, NY**USA
JOURNAL: Blood 98 (11 Part 2):p60b November 16, **2001**
MEDIUM: print
CONFERENCE/MEETING: 43rd Annual Meeting of the American Society of Hematology, Part 2 Orlando, Florida, USA December 07-11, 2001
ISSN: 0006-4971
RECORD TYPE: Abstract
LANGUAGE: English

ABSTRACT: Acute, severe thrombocytopenia is a rare but potentially fatal complication of sarcoidosis. We report a 31 year-old man who presented with epistaxis and severe thrombocytopenia (5 X109/L). **Blood** smear demonstrated normal red and white **blood** cell morphology. **Bone marrow** biopsy was normal. The thrombocytopenia failed to respond to treatments with Rh0 D immune globulin (WinRho), immune globulin (IGIV

and steroids, but responded to splenectomy. Sarcoidosis was found in the spleen of the patient. A literature **review** of an additional 21 cases of acute, severe thrombocytopenia associated with sarcoidosis was performed. Of 22 patients, sixteen patients (16/22) developed bleeding and severe thrombocytopenia prior to the diagnosis of sarcoidosis: eleven (11) presented initially with bleeding and severe thrombocytopenia; 7 presented first with arthralgia, fever, cough or weight loss from 2 weeks to 4 months duration, then thrombocytopenia and bleeding. Thrombocytopenia and bleeding occurred in six (6/22) patients, who were previously diagnosed with sarcoidosis and normal platelet (with the durations of the disease of 1, 6 months and 1, 1, 3, 18 years respectively). All the patients had a **tissue** diagnosis of sarcoidosis eventually. **Bone marrow** biopsy was preformed in all 22 patients and none had evidence of sarcoidosis involvement. Platelet-associated IgGs were detected in all 8 patients in whom it was tested, and Coombs test was positive in 3 of 11 patients tested. Remission was achieved in 12 of 17 patients who were treated with steroids. Of 5 patients who failed steroids, three patients responded to IGIV with steroid, one to vincristine with steroid, and one to splenectomy. Of 5 patients not treated with steroids, three underwent splenectomy with good response. Two patients died of intracranial hemorrhage before effective treatment. Severe thrombocytopenia in sarcoidosis may be mediated by immune mechanisms. Sarcoidosis should be considered in the differential diagnosis of severe thrombocytopenia. Steroids may be offered as the first choice of treatment.

2001

9/3,AB/58 (Item 26 from file: 5)
DIALOG(R)File 5:Biosis Previews(R)

13470137 BIOSIS NO.: 200200098958
Disseminated malignant ectomesenchymoma (MEM): Case report and **review** of the literature.
AUTHOR: Mueller H L(a); Marx A; Trusen M; Schneider P; Kuehl J
AUTHOR ADDRESS: (a)Department of Pediatric Hematology and Oncology, Children's Hospital, Staedtische Kliniken Oldenburg, Cloppenburgerstr. 363, 26133, Oldenburg**Germany E-Mail: mueller.hermann@kliniken-oldenburg.de
JOURNAL: Pediatric Hematology and Oncology 19 (1):p9-17 January-February, **2002**
MEDIUM: print
ISSN: 0888-0018
DOCUMENT TYPE: Article; Literature Review
RECORD TYPE: Abstract
LANGUAGE: English

ABSTRACT: Malignant ectomesenchymoma (MEM) is a rare soft **tissue** tumor believed to arise from a pluripotent migratory neural crest cell and composed of both a mesenchymal element and a neuroectodermal element. The authors report the case of an 11-month-old male who presented with a local abdominal MEM and systemic metastases into lungs, liver, bones, and **bone marrow**. This is the first reported case of an MEM with initial **bone marrow** dissemination. The tumor consisted of a neuroblastoma component and a mesenchymal component with sarcomatous features. Diagnosis and therapy were complicated by the histological heterogeneity of the tumor, which also influenced the clinical appearance and course in this case. A literature search revealed 15 other evaluated cases that arose in soft **tissue** and had adequate clinicopathologic data. Complete surgical resection was the mainstay of treatment, and chemotherapy also appeared to be important. In all reported patients (n = 3) with initial metastases or **bone marrow** dissemination, as

in this case, no cure could be achieved. In patients with disseminated MEM, new therapeutic approaches such as high-dose chemotherapy followed by stem cell rescue should be considered, similar to the current strategy in patients with stage IV neuroblastoma or soft **tissue** sarcoma.

2002

9/3,AB/59 (Item 27 from file: 5)
DIALOG(R)File 5:Biosis Previews(R)

13378932 BIOSIS NO.: 200200007753
Macrophage activation syndrome: A potentially fatal complication of rheumatic disorders.
AUTHOR: Sawhney S; Woo P; Murray K J(a)
AUTHOR ADDRESS: (a)Department of Rheumatology, Great Ormond Street Hospital, London, WC1N 3JH**UK E-Mail: k.murray@ich.ucl.uk
JOURNAL: Archives of Disease in Childhood 85 (5):p421-426 November,
2001
MEDIUM: print
ISSN: 0003-9888
DOCUMENT TYPE: Article
RECORD TYPE: Abstract
LANGUAGE: English

ABSTRACT: Aims: To **review** the precipitating events, clinical features, treatment, and outcome of macrophage activation syndrome (MAS). Methods: Retrospective **review** of cases of MAS from a prospectively collected database of children with rheumatic diseases from 1980 to 2000. Results: Nine patients (eight girls) were considered to have evidence of MAS. The primary diagnosis was systemic onset juvenile idiopathic arthritis in seven, enthesitis related arthritis in one, and chronic infantile neurological cutaneous articular syndrome in one. Mean age of onset was 5.7 years, and duration prior to MAS, 4.2 years. No medication was identified as a trigger. Eight had infections prior to MAS; specific infectious agents were identified in four. High grade fever, new onset hepatosplenomegaly, and lymphadenopathy were common clinical features. Platelet counts fell dramatically, from an average of 346 to 99X109/l. Mean erythrocyte sedimentation rate (in three patients) fell from 115 to 28 mm/h. Eight had abnormal liver function during the disease course, and six had coagulopathy. **Bone marrow** examination supported the diagnosis with definite haemophagocytosis in four of seven. All received high dose steroids (eight intravenous, one oral), five cyclosporin, two cyclophosphamide, and one antithymocyte globulin. Two of three patients with significant renal impairment died. Conclusion: MAS is a rare and potentially fatal complication of childhood rheumatic disorders. Most of our patients were female, and most cases were preceded by infection. **Bone marrow** studies support the diagnosis. Deranged renal function may be a poor prognostic sign. Aggressive early therapy is essential.

2001

9/3,AB/60 (Item 28 from file: 5)
DIALOG(R)File 5:Biosis Previews(R)

13338147 BIOSIS NO.: 200100545296
Cerebellar granulocytic sarcoma in an infant with CD56+ acute monoblastic leukemia.
AUTHOR: Psiachou Leonard Elene(a); Paterakis Georgios; Stefanaki Kalliopi; Mikraki Christou Vasiliki; Haidas Stavros

AUTHOR ADDRESS: a Department of Haematology Oncology, Aghia Sophia Children's Hospital, Thivon and Papadiamanto-poulou, Athens, 11527; epstachou-hotmail.com**Greece
JOURNAL: Leukemia Research 25 (11):p1019-1021 November, **2001**
MEDIUM: print
ISSN: 0145-2126
DOCUMENT TYPE: Article
RECORD TYPE: Abstract
LANGUAGE: English
SUMMARY LANGUAGE: English

ABSTRACT: Granulocytic sarcoma (GS) is a form of extramedullary leukaemia (EML). The presence of the neural cell adhesion molecule (NCAM) on the surface of the blasts, which is recognized by the CD56 monoclonal antibody, enhances their propensity for **tissue** penetration. GS within the central nervous system (CNS), in particular within the cerebellum, is extremely uncommon. We **review** the literature and describe an infant with isolated cerebellar GS relapse, which antedated a CD56+ acute monoblastic leukaemia **bone marrow** (BM) relapse.

2001

9/3,AB/61 (Item 29 from file: 5)
DIALOG(R)File 5:Biosis Previews(R)

13323194 BIOSIS NO.: 200100530343
Hematophagocytic lymphohistiocytosis: **Review** of literature.
AUTHOR: Lukina E A
JOURNAL: Gematologiya i Transfuziologiya 46 (3):p47-50 May-June, **2001**
MEDIUM: print
ISSN: 0234-5730
DOCUMENT TYPE: Literature Review
RECORD TYPE: Citation
LANGUAGE: Russian; Non-English
SUMMARY LANGUAGE: English
2001

9/3,AB/62 (Item 30 from file: 5)
DIALOG(R)File 5:Biosis Previews(R)

13311088 BIOSIS NO.: 200100518237
Sources of hematopoietic stem cells for transplantation.
ORIGINAL LANGUAGE TITLE: Fontes de celulas-tronco hematopoeticas para transplantes.
AUTHOR: Azevedo Wellington(a); Ribeiro Maria Cecilia C
AUTHOR ADDRESS: (a)Av. Brasil, 691/901 - Bairro Sao Lucas, CEP:30.140-000, Belo Horizonte, MG: azevedow@medicina.ufmg.br**Brazil
JOURNAL: Medicina (Ribeirao Preto) 33 (4):p381-389 Outubro-Dezembro, **2000**
MEDIUM: print
ISSN: 0076-6046
DOCUMENT TYPE: Literature Review
RECORD TYPE: Abstract
LANGUAGE: Portuguese; Non-English
SUMMARY LANGUAGE: English; Portuguese

ABSTRACT: Currently, several types of hematopoietic stem cells (HSC), from different tissues and donors, are available for transplantation to human beings. In this **review**, we discuss the use of allogeneic HSC

harvested directly from the **bone marrow** or mobilized to the peripheral **blood** with hematopoietic growth factors. We compared these two types of transplants regarding to engraftment, relapse, acute and chronic graft-versus-host disease and survival. Although there is a clear advantage of PBSCT over BMT in the early posttransplant period, higher frequency of cGVHD in PBSCT and relatively short followup of patients do not recommend generalized adoption of allogeneic HSCT so far.

DESCRIPTORS:
2000

9/3,AB/63 (Item 31 from file: 5)
DIALOG(R)File 5:Biosis Previews(R)

13272274 BIOSIS NO.: 200100479423
Campath-1H (anti-CD52) monoclonal antibody therapy in lymphoproliferative disorders: A **review**.
AUTHOR: Pangalis G A(a); Dimopoulou M N; Angelopoulou M K; Tsekouras Ch; Vassilakopoulos T P; Vaiopoulos G; Siakantaris M P
AUTHOR ADDRESS: (a)1st Department of Internal Medicine, National and Kapodistrian University of Athens School of Medicine, Laikon General Hospital, 17 Agniou Thoma St., Goudi, Athens, 11527: pangalis@otenet.gr** Greece
JOURNAL: Medical Oncology (Totowa) 18 (2):p99-107 **2001**
MEDIUM: print
ISSN: 1357-0560
DOCUMENT TYPE: Literature Review
RECORD TYPE: Abstract
LANGUAGE: English
SUMMARY LANGUAGE: English

ABSTRACT: Campath-1H is a humanized monoclonal antibody targeted against the CDw52 membrane antigen of lymphocytes, which causes complement and antibody-dependent cell-mediated cytotoxicity. Campath-1H has been used in B-chronic lymphocytic leukemia (B-CLL), T-prolymphocytic leukemia (T-PLL), and low-grade non-Hodgkin's lymphoma (LGNHL). Campath-1H is administered intravenously thrice weekly for up to 12 wk, at an initial dose of 3 mg, escalated to 10 and 30 mg. The responses (complete (CR) and partial (PR)) obtained in untreated B-CLL patients are of the order of 90%. In previously treated B-CLL patients, responses are of the order of approximately 40%, with 2-4% CRs. Responses are more prominent in the **blood** and **bone marrow** compared to the lymph nodes. The median duration of response is 9-12 mo. Because of the antibody's higher activity on circulating lymphocytes, it has been used for in vivo purging of residual disease in B-CLL, followed by autologous stem-cell transplantation. In heavily pretreated advanced stage LGNHL, response is achieved only in 14% of cases with B-phenotype; a 50% response rate is noted in mycosis fungoides. In T-PLL, the CR rate is approximately 60%. Promising results have been reported in a small number of patients with refractory autoimmune thrombocytopenia of lymphoproliferative disorders. The main complications of Campath-1H treatment are caused by tumor necrosis factor (TNF)-alpha and interleukin (IL)-6 release, usually during the first intravenous infusion, and include fever, rigor, nausea, vomiting, and hypotension responsive to steroids. These side effects are usually less severe with subsequent infusions and can be prevented by paracetamol and antihistamines. Immunosupression resulting from normal B- and T-lymphocyte depletion is frequent, resulting in an increased risk for opportunistic infections. More clinical trials in a larger number of patients are necessary to determine the exact role and indications of Campath-1H in lymphoproliferative disorders.

2001

9/3,AB/64 (Item 32 from file: 5)
DIALOG(R)File 5:Biosis Previews(R)

13236426 BIOSIS NO.: 200100443575
BK virus: A clinical **review**.
AUTHOR: Reploeg Mark D(a); Storch Gregory A; Clifford David B
AUTHOR ADDRESS: (a)Neurology, Washington University School of Medicine, 660
S. Euclid, Saint Louis, MO, 63110: reploegm@medicine.wustl.edu**USA
JOURNAL: Clinical Infectious Diseases 33 (2):p191-202 15 July, **2001**
MEDIUM: print
ISSN: 1058-4838
DOCUMENT TYPE: Article; Literature Review
RECORD TYPE: Abstract
LANGUAGE: English
SUMMARY LANGUAGE: English

ABSTRACT: We present a **review** of the clinically oriented literature about BK virus, a relative of JC virus, which is the etiologic agent of progressive multifocal leukoencephalopathy (PML). The kidney, lung, eye, liver, and brain have been proposed as sites of BK virus-associated disease, both primary and reactivated. BK virus has also been detected in **tissue** specimens from a variety of neoplasms. We believe that BK virus is most often permissively present in sites of disease in immunosuppressed patients, rather than being an etiologic agent that causes symptoms or pathologic findings. There is, however, strong evidence for BK virus-associated hemorrhagic cystitis and nephritis, especially in recipients of solid organ or **bone marrow** transplants. Now that BK virus can be identified by use of specific and sensitive techniques, careful evaluation of the clinical and pathologic presentations of patients with BK virus will allow us to form a clearer picture of viral-associated pathophysiology in many organ systems.

2001

9/3,AB/65 (Item 33 from file: 5)
DIALOG(R)File 5:Biosis Previews(R)

13205889 BIOSIS NO.: 200100413038
Pathology of autoimmune myelofibrosis: A report of three cases and a **review** of the literature.
AUTHOR: Bass Randall D; Pullarkat Vinod; Feinstein Donald I; Kaul Anita; Winberg Carl D; Brynes Russell K(a)
AUTHOR ADDRESS: (a)Dept of Pathology, USC, 2011 Zonal Ave, HMR 209, Los Angeles, CA, 90033**USA
JOURNAL: American Journal of Clinical Pathology 116 (2):p211-216 August,
2001
MEDIUM: print
ISSN: 0002-9173
DOCUMENT TYPE: Article
RECORD TYPE: Abstract
LANGUAGE: English
SUMMARY LANGUAGE: English

ABSTRACT: We identified 3 patients with autoimmune myelofibrosis (AM) lacking American Rheumatism Association criteria for systemic lupus erythematosus (SLE). They had 1 or 2 cytopenias and lacked serologic evidence for SLE. Autoimmune features included psoriatic arthritis and positive direct Coombs test (DCT) result, DCT-positive autoimmune hemolytic anemia, and synovitis with polyclonal hypergammaglobulinemia.

Bone marrow biopsy specimens from each patient were evaluated by routine morphologic and immunohistochemical examination. They demonstrated marked hypercellularity (2 cases) or hypocellularity (1 case), moderate erythroid hyperplasia (all cases) with left-shifted maturation (2 cases), intrasinusoidal hematopoiesis (all cases), slightly to moderately increased megakaryocytes (2 cases), and grade 3 to 4 reticulin fibrosis (all cases). All lacked basophilia, eosinophilia, bizarre megakaryocytes, clusters of megakaryocytes, and osteosclerosis. Mild to moderate **bone marrow** lymphocytosis was noted in all cases. In 2 cases, increased small T cells and B cells formed nonparatrabecular, loose aggregates. AM is a clinicopathologic entity that may lack features of SLE. Loose aggregates of **bone marrow** T and B lymphocytes and the absence of morphologic and clinical features of myeloproliferative disease or low-grade lymphoproliferative disease are clues that distinguish AM from better known causes of **bone marrow** fibrosis.

2001

9/3,AB/66 (Item 34 from file: 5)
DIALOG(R)File 5:Biosis Previews(R)

13134717 BIOSIS NO.: 200100341866
Tolerance, mixed chimerism and protection against graft-versus-host disease after total lymphoid irradiation.
AUTHOR: Field Elizabeth H; Strober Samuel(a)
AUTHOR ADDRESS: (a)Division of Rheumatology and Immunology, Department of Medicine, Stanford University School of Medicine, Stanford, CA, 94305: sstrober@stanford.edu**USA
JOURNAL: Philosophical Transactions of the Royal Society of London B Biological Sciences 356 (1409):p739-748 29 May, **2001**
MEDIUM: print
ISSN: 0962-8436
DOCUMENT TYPE: Literature Review
RECORD TYPE: Abstract
LANGUAGE: English
SUMMARY LANGUAGE: English

ABSTRACT: Total lymphoid irradiation (TLI), originally developed as a non-myeloablative treatment for Hodgkin's disease, has been adapted for the induction of immune tolerance to organ allografts in rodents, dogs and non-human primates. Moreover, pretransplantation TLI has been used in prospective studies to demonstrate the feasibility of the induction of tolerance to cadaveric kidney allografts in humans. Two types of tolerance, chimeric and non-chimeric, develop after TLI treatment of hosts depending on whether donor **bone marrow** cells are transplanted along with the organ allograft. An advantageous feature of TLI for combined **marrow** and organ transplantation is the protection against graft-versus-host disease (GVHD) and facilitation of chimerism afforded by the predominance of CD4+NK1.1+-like T cells in the irradiated host lymphoid tissues. Recently, a completely post-transplantation TLI regimen has been developed resulting in stable mixed chimerism and tolerance that is enhanced by a brief course of cyclosporine. The post-transplantation protocol is suitable for clinical cadaveric kidney transplantation. This **review** summarizes the evolution of TLI protocols for eventual application to human clinical transplantation and discusses the mechanisms involved in the induction of mixed chimerism and protection from GVHD.

2001

9/3,AB/67 (Item 35 from file: 5)
DIALOG(R)File 5:Biosis Previews(R)

13108697 BIOSIS NO.: 200100335846
The thymus and central tolerance.
AUTHOR: Sprent Jonathan (a); Kishimoto Hidehiro
AUTHOR ADDRESS: (a)Department of Immunology, Scripps Research Institute, 10550 North Torrey Pines Road, IMM4, La Jolla, CA, 92037: jsprent@scripps.edu**USA
JOURNAL: Philosophical Transactions of the Royal Society of London B Biological Sciences 356 (1409):p609-616 29 May, **2001**
MEDIUM: print
ISSN: 0962-8436
DOCUMENT TYPE: Literature Review
RECORD TYPE: Abstract
LANGUAGE: English
SUMMARY LANGUAGE: English

ABSTRACT: T-cell differentiation in the thymus generates a peripheral repertoire of mature T cells that mounts strong responses to foreign antigens but is largely unresponsive to self-antigens. This state of specific immunological tolerance to self-components involves both central and peripheral mechanisms. Here we **review** the process whereby many T cells with potential reactivity for self-antigens are eliminated in the thymus during early T-cell differentiation. This process of central tolerance (negative selection) reflects apoptosis and is a consequence of immature T cells receiving strong intracellular signalling through T-cell receptor (TCR) recognition of peptides bound to major histocompatibility complex (MHC) molecules. Central tolerance occurs mainly in the medullary region of the thymus and depends upon contact with peptide-MHC complexes expressed on **bone marrow**-derived antigen-presenting cells (APCs); whether tolerance also occurs in the cortex is still controversial. Tolerance induction requires a combination of TCR ligation and co-stimulatory signals. Co-stimulation reflects interaction between complementary molecules on T cells and APCs and probably involves multiple molecules acting in concert, which may account for why deletion of individual molecules with known or potential co-stimulatory function has little or no effect on central tolerance. The range of self-antigens that induce central tolerance is considerable and, via low-level expression in the thymus, may also include **tissue**-specific antigens; central tolerance to these latter antigens, however, is likely to be limited to high-affinity T cells, leaving low-affinity cells to escape. Tolerance to alloantigens and the possibility of using central tolerance to promote acceptance of allografts are discussed.

2001

9/3,AB/68 (Item 36 from file: 5)
DIALOG(R)File 5:Biosis Previews(R)

13113512 BIOSIS NO.: 200100320661
Chronic lymphocytic leukemia is significantly more common than reported.
AUTHOR: Zent Clive S(a); Kyasa Mouhammed J(a); King Stephanie L(a); Evans Richard B; Schichman Steven A
AUTHOR ADDRESS: (a)Department of Medicine, Little Rock VAMC and UAMS, Little Rock, AR**USA
JOURNAL: Blood 96 (11 Part 2):p284b November 16, **2000**
MEDIUM: print
CONFERENCE/MEETING: 42nd Annual Meeting of the American Society of Hematology San Francisco, California, USA December 01-05, 2000
SPONSOR: American Society of Hematology

ISSN: 0006-4971
RECORD TYPE: Abstract
LANGUAGE: English
SUMMARY LANGUAGE: English

ABSTRACT: Chronic lymphocytic leukemia (CLL) is frequently diagnosed by flow cytometric analysis of peripheral **blood** lymphocytes. Because Tumor Registry data collection usually requires a **tissue** diagnosis, patients with CLL diagnosed by flow cytometry may fail to be reported to Tumor Registries, which are an important source of epidemiological data. Tumor Registry data may thus significantly underestimate the true incidence of CLL. To test this hypothesis, we reviewed the actual and reported incidence of CLL for 10 years (1990 to 1999) at the Little Rock Veterans Affairs Medical Center (VAMC). **Review** of medical and laboratory records showed that 93 patients had a new diagnosis of CLL. The Tumor Registry reported 60 new cases of CLL from 1990-1999 (65% of actual total). From 1990-1996, when most diagnoses were based on **bone marrow** biopsy, the Tumor Registry reported 91% of newly diagnosed CLL. In 1997, accurate flow cytometry on peripheral **blood** became readily available at the Little Rock VAMC. Increased use of flow cytometry for diagnosis of CLL was associated with a marked decrease in Tumor Registry reporting. From 1997 to 1999, the Tumor Registry reported a significantly lower 22% of patients with newly diagnosed CLL (P < 0.01, Students test). Conclusion: Tumor Registry data significantly underestimated the incidence of CLL at the Little Rock VAMC after 1997 due to the failure to include diagnoses made solely by flow cytometric analysis of peripheral **blood**. Because many hospital Tumor Registries do not routinely monitor flow cytometry results, this may reflect a widespread problem. Our data suggest that the national incidence estimates for CLL based on Tumor Registry data may be low and that the disease may be significantly more common than currently reported. Tumor Registry data collection should be modified to routinely include reports from flow cytometry facilities.

2000

9/3,AB/69 (Item 37 from file: 5)
DIALOG(R)File 5:Biosis Previews(R)

13107659 BIOSIS NO.: 200100314808
Treatment of autoimmune diseases by hematopoietic stem cell transplantation.
AUTHOR: Ikehara Susumu(a)
AUTHOR ADDRESS: (a)First Department of Pathology, Kansai Medical University, 10-15 Fumizono-cho, Moriguchi City, Osaka, 570-8506: ikehara@takii.kmu.ac.jp**Japan
JOURNAL: Experimental Hematology (Charlottesville) 29 (6):p661-669 June, **2001**
MEDIUM: print
ISSN: 0301-472X
DOCUMENT TYPE: Literature Review
RECORD TYPE: Abstract
LANGUAGE: English
SUMMARY LANGUAGE: English

ABSTRACT: Remarkable advances have been made in **bone marrow** transplantation (BMT), which now has become a powerful strategy for the treatment of leukemia, aplastic anemia, congenital immunodeficiency disorders, and autoimmune diseases. Using various animal models, allogeneic BMT has been found to be useful in the treatment of autoimmune diseases. In MRL/lpr mice, which are radiosensitive (8.5 Gy) and are an animal model for autoimmune disorders, conventional BMT resulted in only

transient effects; the manifestations of the autoimmune diseases recurred 3 months after BMT. However, the combination of BMT plus **bone** grafts (to recruit donor stromal cells) was capable of preventing the recurrence of autoimmune diseases in MRL/lpr mice. This strategy was found to be ineffective in the treatment of MRL/lpr mice that had developed autoimmune diseases, because these mice were more sensitive to the effects of radiation after the onset of lupus nephritis due to uremic enterocolitis. We have recently discovered a safer strategy for treatment of autoimmune diseases, which includes fractionated irradiation (5.5 GyX2) (day-1) followed by portal venous injection (day 0) plus intravenous injection (day 5) of donor unfractionated **bone marrow** cells. We successfully treated autoimmune diseases in MRL/lpr mice using this strategy; 100% of MRL/lpr mice treated in this fashion survive >1 year after treatment. We identified the mechanisms underlying the components of this approach and have found that stromal cells play a crucial role in successful BMT. In this **review**, the conditions essential for successful allogeneic BMT are discussed.

2001

9/3,AB/70 (Item 38 from file: 5)
DIALOG(R)File 5:Biosis Previews(R)

13099927 BIOSIS NO.: 200100307076
Primary non-Hodgkin lymphoma of the **bone**: Importance of a multiapproach of imaging.
AUTHOR: Ratterni Sergio(a); Mela Donatella(a); Goretti Riccardo(a); Ardoino Silvia(a); Gandolfo Nicola(a); Artom Patrizia; Artom Alberto(a)
AUTHOR ADDRESS: (a)Departments of Medicine, Pathology and Radiology, Santa Corona Hospital, Pietra Ligure**Italy
JOURNAL: Blood 96 (11 Part 2):p230b November 16, **2000**
MEDIUM: print
CONFERENCE/MEETING: 42nd Annual Meeting of the American Society of Hematology San Francisco, California, USA December 01-05, 2000
SPONSOR: American Society of Hematology
ISSN: 0006-4971
RECORD TYPE: Abstract
LANGUAGE: English
SUMMARY LANGUAGE: English

ABSTRACT: To **review** the role of each imaging modality in the diagnosis, staging, follow-up during combined chemioradiotherapy and detection of recurrences of primary NH Lymphoma of the **bone**, we have prospectively studied 8 patients (5 female, 3 male; age 19-79, mean 51) with biopsy-proven primary NH Lymphoma without evidence of systemic disease. All have been studied with plain films and CT; MR was obtained in 6/8 cases. US was obtained to evaluate soft **tissue** involvement in all cases. Percutaneous needle biopsy (6 bones, 2 soft **tissue** masses) was always performed. Radiological follow-up after combined chemioradiotherapy was performed in all cases. In five patients the primary NH Lymphoma affected the axial skeleton, in two the iliac **bone** and in one the femur. The most common pathologic type was follicular lymphoma (B-cell). The radiographic appearance ranged from lytic(n=5 pts) to sclerotic. CT showed respectively the lytic character and focal cortical change. MR showed a focal area of low signal intensity on T1W, high intensity on 2TW and STIR, with marked contrast-medium uptake. US identified a non homogeneous solid mass involving the soft tissues, with loss of the osseous integrity. MR better evaluated the neoplastic extension in the **bone marrow** and surrounding extraosseous structures, before and following multimodality therapy. The diagnosis of primary NH Lymphoma of the **bone** is made on the basis of histopathological examination of the biopsy of the affected **bone**

. Clinical and hematological data can be helpful in staging the disease. As shown in our cases, radiological imaging are absolutely determinant in the processing of diagnosis. The various radiological imaging techniques are complementary, rather than competitive, for complete assessment of the disease both at diagnosis and in follow-up.

2000

9/3,AB/71 (Item 39 from file: 5)
DIALOG(R)File 5:Biosis Previews(R)

13193010 BIOSIS NO.: 200100305159
Granulocytic sarcoma in a patient with hemoglobin SC disease.
AUTHOR: Jain Hitender(a); Zacharais Leo(a); Ballas Samir K; Gay Roy(a)
AUTHOR ADDRESS: (a)Department of Internal Medicine, Mercy Hospital of Philadelphia, Philadelphia, PA**USA
JOURNAL: Blood 96 (11 Part 2):p18b November 16, **2000**
MEDIUM: print
CONFERENCE/MEETING: 42nd Annual Meeting of the American Society of Hematology San Francisco, California, USA December 01-05, 2000
SPONSOR: American Society of Hematology
ISSN: 0006-4971
RECORD TYPE: Abstract
LANGUAGE: English
SUMMARY LANGUAGE: English

ABSTRACT: We present a rare case of and granulocytic sarcoma in a patient with sickle cell disease (SCD). The patient is a 38-year old man with known Hemoglobin SC disease who sought medical attention because of severe pain and swelling in his left gluteal region and left thigh. At presentation his WBC count was 352 thousand/microliter with 48 % polychromatophils, hemoglobin of 9.2 gm/dl and a platelet count of 975 thousand/microliter. Multiple computed tomographic scans of the lower extremities were performed, which showed marked necrosis in his gluteal and quadriceps group of muscles on the left side. He subsequently underwent an open debridement. Histopathology report from the excised **tissue** showed a differentiated granulocytic sarcoma. A **bone marrow** biopsy showed nearly 100% cellularity with trilineage hyperplasia, which was more pronounced in the granulocytic line. There was a myelocytic bulge and blasts constituted less than 1% of all nucleated cells. The patient subsequently received local irradiation and was placed on hydroxyurea at a dosage of 1500 mg/day. He showed clinical resolution of the pain and swelling as well as normalization of his WBC and platelet count. Upon **review** of the medical literature this appears to be the first case report of a patient with Hemoglobin SC disease presenting with granulocytic sarcoma.

2000

9/3,AB/72 (Item 40 from file: 5)
DIALOG(R)File 5:Biosis Previews(R)

12960045 BIOSIS NO.: 200100167194
Mesenchymal stem cells: Heading into the clinic.
AUTHOR: Koc O N(a); Lazarus H M
AUTHOR ADDRESS: (a)Ireland Cancer Center, University Hospitals of Cleveland, Department of Medicine/Division of Hematology Oncology, Case Western Reserve University, 10900 Euclid Avenue, Cleveland, OH, 44106** USA
JOURNAL: Bone Marrow Transplantation 27 (3):p235-239 February 1,

2001
MEDIUM: print
ISSN: 0268-3369
DOCUMENT TYPE: Literature Review
RECORD TYPE: Abstract
LANGUAGE: English
SUMMARY LANGUAGE: English

ABSTRACT: In recent years, there has been an increasing interest in non-hematopoietic pluripotent progenitor cells that are found in the **bone marrow**. Mesenchymal stem cells (MSCs) are the first non-hematopoietic progenitors to be isolated from the **bone marrow** and extensively characterized. In addition to their ability to support hematopoiesis, MSCs can differentiate into osteocytes, chondrocytes, tenocytes, adipocytes and smooth muscle cells. This article will **review** our current understanding of **bone marrow** stroma and MSCs and their potential therapeutic role in the setting of hematopoietic stem cell transplantation.

2001

9/3,AB/73 (Item 41 from file: 5)
DIALOG(R)File 5:Biosis Previews(R)

12877927 BIOSIS NO.: 200100085076
Ex vivo expansion of human hematopoietic progenitors and cells to support high-dose chemoradiation therapy: Five years of clinical experience.
AUTHOR: Chabannon Christian(a); Olivero Sylvian; Blaise Didier; Maraninchi Dominique; Viens Patrice
AUTHOR ADDRESS: (a)Centre de Therapie Cellulaire, Institut Paoli-Calmettes, 232 boulevard Sainte Marguerite, 13273, Marseille Cedex, 9: chabannonc@marseille.fnclcc.fr**France
JOURNAL: Cytokines Cellular & Molecular Therapy 6 (2):p97-108 June,
2000
MEDIUM: print
ISSN: 1368-4736
DOCUMENT TYPE: Literature Review
RECORD TYPE: Abstract
LANGUAGE: English
SUMMARY LANGUAGE: English

ABSTRACT: The identification of cytokines-soluble or membrane-bound regulators of hematopoietic stem and progenitor cell survival, proliferation, and differentiation - and the definition of culture conditions that enable cell and progenitor expansion, has lead to the first clinical trials using cultured cells in addition to or in place of unmanipulated cells. The use of ex vivo expanded cells can improve several aspects of autologous and allogeneic hematopoietic cell and progenitor transplantation, such as reducing or abolishing the nadir that follows high-dose chemoradiation therapy regimens, or reducing the clinical risks associated with the use of small numbers of progenitors as in cord **blood** transplantation and in autologous transplantation for poor mobilizers. In addition, biological questions raised by ex vivo expansion are shared by scientists and clinicians interested in gene transfer into hematopoietic stem cells. We here **review** the biological problems associated with ex vivo expansion: defining efficient culture conditions, considering not only scientific and biological issues but also regulatory and commercial issues, defining appropriate surrogate endpoints that predict engraftment and superior clinical efficacy to that obtained with the use of unmanipulated grafts. We also **review** the results of the first clinical trials that have demonstrated the feasibility of this approach, and have shown some of its limitations;

demonstration of clinical efficacy will require more preclinical and clinical work.

2000

9/3,AB/74 (Item 42 from file: 5)
DIALOG(R)File 5:Biosis Previews(R)

12802047 BIOSIS NO.: 200100009196
Has the outlook improved for amifostine as a clinical radioprotector?
AUTHOR: Lindegaard Jacob Christian(a); Grau Cai
AUTHOR ADDRESS: (a)Department of Oncology, Aarhus University Hospital, Norrebrogade 44, DK-8000, Aarhus C**Denmark
JOURNAL: Radiotherapy & Oncology 57 (2):p113-118 November, **2000**
MEDIUM: print
ISSN: 0167-8140
DOCUMENT TYPE: Literature Review
RECORD TYPE: Abstract
LANGUAGE: English
SUMMARY LANGUAGE: English

ABSTRACT: Amifostine has recently been approved for clinical radiotherapy as a protector against irradiation-induced xerostomia. It is our aim to **review** the outlook for using amifostine as a general clinical radioprotector. Protection against X-rays is mainly obtained by the scavenging of free radicals. The degree of protection is therefore highly dependent on oxygen tension, with protection factors ranging from 1 to 3. Maximal protection is observed at physiological levels of oxygenation. A great variability in protection has also been observed between different normal tissues. Some **tissue**, like brain, is not protected while salivary glands and **bone marrow** may exhibit a three-fold increase in radiation tolerance. Amifostine is dephosphorylized to its active metabolite by a process involving alkaline phosphatase. Due to lower levels of alkaline phosphatase in tumor vessels, amifostine is marketed as a selective protector of normal **tissue** and not tumors. However, the preclinical investigations concerning the selectivity of amifostine are controversial and the clinical studies are sparse and do not have the power to evaluate the influence of amifostine on the therapeutic index. Conclusion: based on the present knowledge amifostine should only be used in experimental protocols and not in routine practice.

2000

9/3,AB/75 (Item 43 from file: 5)
DIALOG(R)File 5:Biosis Previews(R)

12692110 BIOSIS NO.: 200000445612
Magnetic resonance imaging in early inflammatory arthritis: What is its role?.
AUTHOR: McQueen F M(a)
AUTHOR ADDRESS: (a)Department of Rheumatology, Auckland Hospital, Auckland 1**New Zealand
JOURNAL: Rheumatology (Oxford) 39 (7):p700-706 July, **2000**
MEDIUM: print
ISSN: 1462-0324
DOCUMENT TYPE: Literature Review
RECORD TYPE: Abstract
LANGUAGE: English
SUMMARY LANGUAGE: English

ABSTRACT: Magnetic resonance imaging MRI has important applications in musculoskeletal medicine. It allows the visualization of **bone** and soft tissues in three dimensions using a multiplanar technique and is uniquely suited to imaging the rheumatoid joint. Bony erosions are seen well using MRI in early rheumatoid arthritis and are frequently detected before they appear on plain radiographs. **Bone marrow** oedema is another important MRI feature associated with inflammatory joint disease and may be a forerunner of erosion. Synovial membrane inflammation and hypertrophy are detected after contrast enhancement and also by the use of dynamic MRI techniques, which provide a non-invasive method to accurately measure the inflammatory process. This information can be analysed and collated using MRI scoring systems and ultimately may be used to improve diagnostic accuracy, predict prognosis and monitor therapy in these patients. This **review** examines the case for the use of MRI in early inflammatory arthritis, outlining its strengths and potential weaknesses as an imaging modality in this context and indicating its potential role in clinical practice.

2000

9/3,AB/76 (Item 44 from file: 5)
DIALOG(R)File 5:Biosis Previews(R)

12658017 BIOSIS NO.: 200000411519
Giant adrenal myelolipoma: Case report and **review** of the literature.
AUTHOR: Yildiz Levent(a); Akpolat Ilkser; Erzurumlu Kenan; Aydin Oguz; Kandemir Bedri
AUTHOR ADDRESS: (a)Department of Pathology, Ondokuz Mayis University School of Medicine, Samsun, 55139**Turkey
JOURNAL: Pathology International 50 (6):p502-504 June, **2000**
MEDIUM: print
ISSN: 1320-5463
DOCUMENT TYPE: Article
RECORD TYPE: Abstract
LANGUAGE: English
SUMMARY LANGUAGE: English

ABSTRACT: Myelolipoma is a tumor-like growth composed of mature fat **tissue** and **bone marrow** elements. It occurs in the adrenal gland or as an isolated soft **tissue** mass. It may be associated with endocrine disorders such as hermaphroditism, Cushing's disease, Addison's disease and obesity of unknown cause. These lesions rarely measure more than 5 cm in diameter, although giant tumors have been reported in the literature. The fifth largest surgically resected adrenal myelolipoma in the literature is reported and its clinical associations and, macroscopic and microscopic features are discussed.

2000

9/3,AB/77 (Item 45 from file: 5)
DIALOG(R)File 5:Biosis Previews(R)

12650770 BIOSIS NO.: 200000404272
Canine models for human genetic neurodegenerative diseases.
AUTHOR: Barsoum Suzy C; Callahan Heather M; Robinson Kelly; Chang Patricia L(a)
AUTHOR ADDRESS: (a)Department of Pediatrics Health Sciences Centre, McMaster University, 1200 Main Street West, Room 3N18, Hamilton, ON, L8S 4J9**Canada

JOURNAL: Progress in Neuro-Psychopharmacology & Biological Psychiatry 24 5 :p811-823 July, **2000**
MEDIUM: print
ISSN: 0278-5846
DOCUMENT TYPE: Literature Review
RECORD TYPE: Abstract
LANGUAGE: English
SUMMARY LANGUAGE: English

ABSTRACT: 1. Canine models of human neurodegenerative disorders are uncommon. However, the similarity between canines and humans in body sizes and physiology provides an exceptional opportunity to use these models to study human diseases. 2. The authors will present a **review** on the neurological deficits that have been observed in canine models of genetic neurodegenerative diseases, and summarize the current gene therapy treatments being developed for some of these conditions.

2000

9/3,AB/78 (Item 46 from file: 5)
DIALOG(R)File 5:Biosis Previews(R)

12626232 BIOSIS NO.: 200000379734
Hemophagocytic syndrome as an unusual form of presentation of tuberculosis in a hemodialysis patient: Case report and **review** of the literature.
AUTHOR: Castellano I(a); Gomez-Martino J R; Hernandez T; Mateos L; Arguello C
AUTHOR ADDRESS: (a)Division of Nephrology, Hospital San Pedro de Alcantara, Avda Millan Astray s/n, E-10003, Caceres**Spain
JOURNAL: American Journal of Nephrology 20 (3):p214-216 May-June, **2000**
MEDIUM: print
ISSN: 0250-8095
DOCUMENT TYPE: Article
RECORD TYPE: Abstract
LANGUAGE: English
SUMMARY LANGUAGE: English

ABSTRACT: We present an unusual manifestation of tuberculosis in a patient on hemodialysis. A 73-year-old woman was admitted to our hospital with a picture of fever, dyspnea and weight loss. She had chronic renal failure and had started periodic hemodialysis 5 years before. Fifteen days after admission, she began with pancytopenia, abnormal liver function and coagulopathy. A **bone marrow** aspiration was made 1 week later showing macrophage elements with phagocytic activity. Eight weeks later, **bone marrow** culture in Lowenstein media confirmed the presence of tuberculosis. After the beginning of antituberculosis therapy, the laboratory disturbances disappeared and the clinical situation improved. We think that fever of unknown origin and pancytopenia in patients on maintenance hemodialysis must lead to an early **bone marrow** biopsy or aspiration since after the diagnosis a specific therapy can cure the disease.

2000

9/3,AB/79 (Item 47 from file: 5)
DIALOG(R)File 5:Biosis Previews(R)

12620372 BIOSIS NO.: 200000373874
Transplantation into genetically alymphoid mice as an approach to dissect the roles of uterine natural killer cells during pregnancy: A **review**.
AUTHOR: Croy B A a ; Di Santo J P; Greenwood J D; Chantakru S; Ashkar A A
AUTHOR ADDRESS: a Department of Biomedical Sciences, University of Guelph, Guelph, Ontario, N1G 2W1**Canada
JOURNAL: Placenta 21 (Suppl. A):pS77-S80 March-April, **2000**
MEDIUM: print
ISSN: 0143-4004
DOCUMENT TYPE: Literature Review
RECORD TYPE: Abstract
LANGUAGE: English
SUMMARY LANGUAGE: English

ABSTRACT: Mice genetically deficient in the natural killer (NK) cell lineage lack uterine (uNK cells) and demonstrate morphometrically-quantifiable histopathology within their implantation sites. Two particular mouse strains, tgepsilon,26 and RAG-2 null X gammac null, have been used successfully as transplant recipients to address questions relating to the biology of uNK cells. uNK cells did not differentiate within decidualized uterine graft segments from normal mice, which were anastomosed orthotopically into immunodeficient hosts. uNK cells did appear in similar grafts placed into immunocompetent hosts, indicating that uNK cells or their progenitors must home to the uterus. This was confirmed by splenocyte transplantation into pregnant uNK cell deficient recipients. Only splenocytes from pregnant donors, not those from non-pregnant donors, homed to the uterus. Homing in this in vivo assay was independent of the CC-chemokine receptors, CCR-2 and CCR-5. Longer-term **bone marrow** cell reconstitution of neonatal or virgin adult uNK cell-deficient mice has identified a functional role for uNK cells in modification of the decidual arterioles which is mediated by IFN-gamma. By utilizing mutant and gene-ablated mice as donors for **tissue** or haematopoietic cell transplants to uNK cell deficient mice, it should be possible to fully characterize the in vivo regulation and functions of these pregnancy-specific uterine lymphocytes.

2000

9/3,AB/80 (Item 48 from file: 5)
DIALOG(R)File 5:Biosis Previews(R)

12501559 BIOSIS NO.: 200000255061
Stem cell transplantation in experimental models of autoimmune disease.
AUTHOR: van Bekkum Dirk W(a)
AUTHOR ADDRESS: (a)Introgene BV, 2301 CA, Leiden**Netherlands
JOURNAL: Journal of Clinical Immunology 20 (1):p10-16 Jan., **2000**
MEDIUM: print
ISSN: 0271-9142
DOCUMENT TYPE: Article
RECORD TYPE: Abstract
LANGUAGE: English
SUMMARY LANGUAGE: English

ABSTRACT: A **review** of the experiments with animal models of autoimmune disease (AID) that have provided the rationale for the present clinical investigations on the use of autologous stem cells for treating patients with severe refractory AID. The various types of AID in laboratory animals and the recognition of the key-role of hematopoitic stem cells (HSC) in AID are discussed. Two animal models were employed for translational research on autologous **bone marrow** transplantation (BMT): adjuvant arthritis (AA) as model for rheumatoid

arthritis RA and experimental allergic encephalomyelitis EAE as model for multiple sclerosis MS . The principal aspects of the treatment, i.e., conditioning agents and doses and T cell depletion of the autograft, were investigated in relation to remission induction and the incidence of relapses.

2000

9/3,AB/81 (Item 49 from file: 5)
DIALOG(R)File 5:Biosis Previews(R)

12474026 BIOSIS NO.: 200000227528
Gene transfer to suppress **bone marrow** alkylation sensitivity.
AUTHOR: Roth Richard B; Samson Leona D(a)
AUTHOR ADDRESS: (a)Department of Cancer Cell Biology, Harvard School of Public Health, 665 Huntington Avenue, Boston, MA, 02115**USA
JOURNAL: Mutation Research 462 (2-3):p107-120 April, **2000**
ISSN: 0027-5107
DOCUMENT TYPE: Article
RECORD TYPE: Abstract
LANGUAGE: English
SUMMARY LANGUAGE: English

ABSTRACT: Alkylating agents represent a highly cytotoxic class of chemotherapeutic compounds that are extremely effective anti-tumor agents. Unfortunately, alkylating agents damage both malignant and non-malignant tissues. **Bone marrow** is especially sensitive to damage by alkylating agent chemotherapy, and is a dose-limiting **tissue** when treating cancer patients. One strategy to overcome **bone marrow** sensitivity to alkylating agent exposure involves gene transfer of the DNA repair protein O6-methylguanine DNA methyltransferase (O6MeG DNA MTase) into **bone marrow** cells. O6MeG DNA MTase is of particular interest because it functions to protect against the mutagenic, clastogenic and cytotoxic effects of many chemotherapeutic alkylating agents. By increasing the O6MeG DNA MTase repair capacity of **bone marrow** cells, it is hoped that this **tissue** will become alkylation resistant, thereby increasing the therapeutic window for the selective destruction of malignant **tissue**. In this **review**, the field of O6MeG DNA MTase gene transfer into **bone marrow** cells will be summarized with an emphasis placed on strategies used for suppressing the deleterious side effects of chemotherapeutic alkylating agent treatment.

2000

9/3,AB/82 (Item 1 from file: 444)
DIALOG(R)File 444:New England Journal of Med.

00123351

Current Concepts: How Contagious Is Vaccinia? (**Review** Article)

Sepkowitz, Kent A.
The New England Journal of Medicine
Jan 30, **2003**; 348 (5),pp 439-446
LINE COUNT: 00321 WORD COUNT: 04438

9/3,AB/83 (Item 2 from file: 444)

DIALOG(R)File 444:New England Journal of Med.

00123253

Easy to See but Hard to Find Clinical Problem-Solving

Reilly, Brendan M.; Clarke, Peter; Nikolinakos, Petros.
The New England Journal of Medicine
Jan 2, **2003**; 348 (1),pp 59-64
LINE COUNT: 00345 WORD COUNT: 04774

9/3,AB/84 (Item 3 from file: 444)
DIALOG(R)File 444:New England Journal of Med.

00123134

Weekly Clinicopathological Exercises: Case 37-2002: A 69-Year-Old Man with Painful Cutaneous Nodules, Elevated Lipase Levels, and Abnormal Results on Abdominal Scanning (Case Records of the Massachusetts General Hospital)

Ashley, Stanley W.; Lauwers, Gregory Y.
The New England Journal of Medicine
Nov 28, **2002**; 347 (22),pp 1783-1791
LINE COUNT: 00449 WORD COUNT: 06207

9/3,AB/85 (Item 4 from file: 444)
DIALOG(R)File 444:New England Journal of Med.

00123132

Medical Progress: Typhoid Fever (**Review** Article)

Parry, Christopher M.; Hien, Tran Tinh; Dougan, Gordon; White, Nicholas J.; Farrar, Jeremy J.
The New England Journal of Medicine
Nov 28, **2002**; 347 (22),pp 1770-1782
LINE COUNT: 00651 WORD COUNT: 08992

9/3,AB/86 (Item 5 from file: 444)
DIALOG(R)File 444:New England Journal of Med.

00123084

Weekly Clinicopathological Exercises: Case 35-2002: R. Michael Scott and E. Tessa Hedley-Whyte (Case Records of the Massachusetts General Hospital)

Scott, R. Michael; Hedley-Whyte, E. Tessa.
The New England Journal of Medicine
Nov 14, **2002**; 347 (20),pp 1604-1611
LINE COUNT: 00507 WORD COUNT: 07005

9/3,AB/87 (Item 6 from file: 444)

DIALOG(R)File 444:New England Journal of Med.

00122933

Drug Therapy: Analgesics for the Treatment of Pain in Children
Review Article

Berde, Charles B.; Sethna, Navil F.
The New England Journal of Medicine
Oct 3, **2002**; 347 (14),pp 1094-1103
LINE COUNT: 00501 WORD COUNT: 06920

9/3,AB/88 (Item 7 from file: 444)
DIALOG(R)File 444:New England Journal of Med.

00122834

Out of Africa (Clinical Problem-Solving)

Sahlas, Demetrios J.; MacLean, J. Dick; Janevski, John; Detsky, Allan S.
The New England Journal of Medicine
Sep 5, **2002**; 347 (10),pp 749-753
LINE COUNT: 00383 WORD COUNT: 05295

9/3,AB/89 (Item 8 from file: 444)
DIALOG(R)File 444:New England Journal of Med.

00122771

Mechanisms of Disease: Thrombotic Microangiopathies (**Review** Article)

Moake, Joel L.
The New England Journal of Medicine
Aug 22, **2002**; 347 (8),pp 589-600
LINE COUNT: 00536 WORD COUNT: 07404

9/3,AB/90 (Item 9 from file: 444)
DIALOG(R)File 444:New England Journal of Med.

00122740

Efficacy and Safety of Imatinib Mesylate in Advanced Gastrointestinal Stromal Tumors (Original Articles)

Demetri, George D.; von Mehren, Margaret; Blanke, Charles D.; Van den Abbeele, Annick D.; Eisenberg, Burton; Roberts, Peter J.; Heinrich, Michael C.; Tuveson, David A.; Singer, Samuel; Janicek, Milos; Fletcher, Jonathan A.; Silverman, Stuart G.; Silberman, Sandra L.; Capdeville, Renaud; Kiese, Beate; Peng, Bin; Dimitrijevic, Sasa; Druker, Brian J.; Corless, Christopher; Fletcher, Christopher D.M.; Joensuu, Heikki.
The New England Journal of Medicine

Aug 15, **2002**; 347 (7),pp 472-480
LINE COUNT: 00368 WORD COUNT: 05090

Abstract

Background: Constitutive activation of KIT receptor tyrosine kinase is critical in the pathogenesis of gastrointestinal stromal tumors. Imatinib mesylate, a selective tyrosine kinase inhibitor, has been shown in preclinical models and preliminary clinical studies to have activity against such tumors.

Methods: We conducted an open-label, randomized, multicenter trial to evaluate the activity of imatinib in patients with advanced gastrointestinal stromal tumor. We assessed antitumor response and the safety and tolerability of the drug. Pharmacokinetics were assessed in a subgroup of patients.

Results: A total of 147 patients were randomly assigned to receive 400 mg or 600 mg of imatinib daily. Overall, 79 patients (53.7 percent) had a partial response, 41 patients (27.9 percent) had stable disease, and for technical reasons, response could not be evaluated in 7 patients (4.8 percent). No patient had a complete response to the treatment. The median duration of response had not been reached after a median follow-up of 24 weeks after the onset of response. Early resistance to imatinib was noted in 20 patients (13.6 percent). Therapy was well tolerated, although mild-to-moderate edema, diarrhea, and fatigue were common. Gastrointestinal or intraabdominal hemorrhage occurred in approximately 5 percent of patients. There were no significant differences in toxic effects or response between the two doses. Imatinib was well absorbed, with pharmacokinetics similar to those reported in patients with chronic myeloid leukemia.

Conclusions: Imatinib induced a sustained objective response in more than half of patients with an advanced unresectable or metastatic gastrointestinal stromal tumor. Inhibition of the KIT signal-transduction pathway is a promising treatment for advanced gastrointestinal stromal tumors, which resist conventional chemotherapy. (N Engl J Med 2002;347:472-80.)

9/3,AB/91 (Item 10 from file: 444)
DIALOG(R)File 444:New England Journal of Med.

00122727

Medical Progress: Inflammatory Bowel Disease (**Review** Article)

Podolsky, Daniel K.
The New England Journal of Medicine
Aug 8, **2002**; 347 (6),pp 417-429
LINE COUNT: 00553 WORD COUNT: 07633

9/3,AB/92 (Item 11 from file: 444)
DIALOG(R)File 444:New England Journal of Med.

00122685

Weekly Clinicopathological Exercises: Case 22-2002: A 37-Year-Old Man with Unexplained Fever after a Long Trip through South America (Case Records of the Massachusetts General Hospital)

Noble, James T.; Mark, Eugene J.
The New England Journal of Medicine

Jul 18, **2002**; 347 (3),pp 200-206
LINE COUNT: 00413 WORD COUNT: 05703

9/3,AB/93 (Item 12 from file: 444)
DIALOG(R)File 444:New England Journal of Med.

00122674

Medical Progress: Cystinosis (**Review** Article)

Gahl, William A.; Thoene, Jess G.; Schneider, Jerry A.
The New England Journal of Medicine
Jul 11, **2002**; 347 (2),pp 111-121
LINE COUNT: 00476 WORD COUNT: 06572

9/3,AB/94 (Item 13 from file: 444)
DIALOG(R)File 444:New England Journal of Med.

00122652

Long-Term Care after Hematopoietic-Cell Transplantation in Adults (Clinical Practice)

Antin, Joseph H.
The New England Journal of Medicine
Jul 4, **2002**; 347 (1),pp 36-42
LINE COUNT: 00267 WORD COUNT: 03698

9/3,AB/95 (Item 14 from file: 444)
DIALOG(R)File 444:New England Journal of Med.

00122503

Weekly Clinicopathological Exercises: Case 19-2002: A 13-Year-Old Girl with a Mass in the Left Parotid Gland and Regional Lymph Nodes (Case Records of the Massachusetts General Hospital)

McGill, Trevor J.I.; Wu, Chin-Lee.
The New England Journal of Medicine
Jun 20, **2002**; 346 (25),pp 1989-1996
LINE COUNT: 00504 WORD COUNT: 06963

9/3,AB/96 (Item 15 from file: 444)
DIALOG(R)File 444:New England Journal of Med.

00122497

Current Concepts: Major Radiation Exposure -- What to Expect and How to Respond (**Review** Article)

Mettler, Fred A., Jr.; Voelz, George L.
The New England Journal of Medicine

May 16, **2002**; 346 (20),pp 1554-1561
LINE COUNT: 00433 WORD COUNT: 05977

9/3,AB/97 (Item 16 from file: 444)
DIALOG(R)File 444:New England Journal of Med.

00122495

Brief Report: Disputed Maternity Leading to Identification of Tetragametic Chimerism (Original Articles)

Yu, Neng; Kruskall, Margot S.; Yunis, Juan J.; Knoll, Joan H.M.; Uhl, Lynne; Alosco, Sharon; Ohashi, Marina; Clavijo, Olga; Husain, Zaheed; Yunis, Emilio J.; Yunis, Jorge J.; Yunis, Edmond J.
The New England Journal of Medicine
May 16, **2002**; 346 (20),pp 1545-1552
LINE COUNT: 00344 WORD COUNT: 04753

9/3,AB/98 (Item 17 from file: 444)
DIALOG(R)File 444:New England Journal of Med.

00122494

Weekly Clinicopathological Exercises: Case 15-2002: A 53-Year-Old Man with a Myocardial Infarct and Thromboses after Coronary-Artery Bypass Grafting (Case Records of the Massachusetts General Hospital)

Aird, William C.; Mark, Eugene J.
The New England Journal of Medicine
May 16, **2002**; 346 (20),pp 1562-1570
LINE COUNT: 00457 WORD COUNT: 06310

9/3,AB/99 (Item 18 from file: 444)
DIALOG(R)File 444:New England Journal of Med.

00122484

Stem Cells -- Scientific, Medical, and Political Issues (Sounding Board)

Weissman, Irving L.
The New England Journal of Medicine
May 16, **2002**; 346 (20),pp 1576-1579
LINE COUNT: 00267 WORD COUNT: 03685

9/3,AB/100 (Item 19 from file: 444)
DIALOG(R)File 444:New England Journal of Med.

00122338

Medical Progress: Immune Thrombocytopenic Purpura (**Review** Article)

Cines, Douglas B.; Blanchette, Victor S.

The New England Journal of Medicine
Mar 28, **2002**; 346 (13),pp 995-1008
LINE COUNT: 00690   WORD COUNT: 09527

9/3,AB/101   (Item 20 from file: 444)
DIALOG(R)File 444:New England Journal of Med.

00122266

Can Human Hematopoietic Stem Cells Become Skin, Gut, or Liver Cells? (Editorial)

Abkowitz, Janis L.
The New England Journal of Medicine
Mar 7, **2002**; 346 (10),pp 770-772
LINE COUNT: 00127   WORD COUNT: 01757

9/3,AB/102   (Item 21 from file: 444)
DIALOG(R)File 444:New England Journal of Med.

00122262

Hepatocytes and Epithelial Cells of Donor Origin in Recipients of Peripheral-**Blood** Stem Cells (Original Articles)

Korbling, Martin; Katz, Ruth L.; Khanna, Abha; Ruifrok, Arnout C.; Rondon, Gabriela; Albitar, Maher; Champlin, Richard E.; Estrov, Zeev.
The New England Journal of Medicine
Mar 7, **2002**; 346 (10),pp 738-746
LINE COUNT: 00389   WORD COUNT: 05371

Abstract
Background: **Bone marrow** contains stem cells with the potential to differentiate into mature cells of various organs. We determined whether circulating stem cells have a similar potential.
Methods: Biopsy specimens from the liver, gastrointestinal tract, and skin were obtained from 12 patients who had undergone transplantation of hematopoietic stem cells from peripheral **blood** (11 patients) or **bone marrow** (1 patient). Six female patients had received transplants from a male donor. Five had received a sex-matched transplant, and one had received an autologous transplant. Hematopoietic stem-cell engraftment was verified by cytogenetic analysis or restriction-fragment-length polymorphism analysis. The biopsies were studied for the presence of donor-derived epithelial cells or hepatocytes with the use of fluorescence in situ hybridization of interphase nuclei and immunohistochemical staining for cytokeratin, CD45 (leukocyte common antigen), and a hepatocyte-specific antigen.
Results: All six recipients of sex-mismatched transplants showed evidence of complete hematopoietic donor chimerism. XY-positive epithelial cells or hepatocytes accounted for 0 to 7 percent of the cells in histologic sections of the biopsy specimens. These cells were detected in liver **tissue** as early as day 13 and in skin **tissue** as late as day 354 after the transplantation of peripheral-**blood** stem cells. The presence of donor cells in the biopsy specimens did not seem to depend on the intensity of **tissue** damage induced by graft-versus-host disease.
Conclusions: Circulating stem cells can differentiate into mature hepatocytes and epithelial cells of the skin and gastrointestinal tract. (N Engl J Med 2002;346:738-46.)

9/3,AB/103 (Item 22 from file: 444)
DIALOG(R)File 444:New England Journal of Med.

00122216

Weekly Clinicopathological Exercises: Case 6-2002: A 54-Year-Old Woman with Left, Then Right, Central-Retinal-Vein Occlusion (Case Records of the Massachusetts General Hospital)

Weinstein, Robert; Mahmood, Mohammad.
The New England Journal of Medicine
Feb 21, **2002**; 346 (8),pp 603-610
LINE COUNT: 00408 WORD COUNT: 05636

9/3,AB/104 (Item 23 from file: 444)
DIALOG(R)File 444:New England Journal of Med.

00122160

Weekly Clinicopathological Exercises: Case 4-2002: A 75-Year-Old Man with Acute Renal Failure Five Months after Cystoprostatectomy and Urethrectomy for Carcinoma (Case Records of the Massachusetts General Hospital)

Bazari, Hasan; Mauiyyedi, Shamila.
The New England Journal of Medicine
Jan 31, **2002**; 346 (5),pp 353-360
LINE COUNT: 00427 WORD COUNT: 05902

9/3,AB/105 (Item 24 from file: 444)
DIALOG(R)File 444:New England Journal of Med.

00122140

Mechanisms of Disease: Production and Actions of Estrogens (**Review** Article)

Gruber, Christian J.; Tschugguel, Walter; Schneeberger, Christian; Huber, Johannes C.
The New England Journal of Medicine
Jan 31, **2002**; 346 (5),pp 340-352
LINE COUNT: 00466 WORD COUNT: 06443

9/3,AB/106 (Item 25 from file: 444)
DIALOG(R)File 444:New England Journal of Med.

00122026

Major-Histocompatibility-Complex Class I Alleles and Antigens in Hematopoietic Cell Transplantation (Original Articles)

Petersdorf, Effie W.; Hansen, John A.; Martin, Paul J.; Woolfrey, Ann;

Malkki, Mari; Gooley, Theodore; Storer, Barry; Mickelson, Eric;
Smith, Anajane; Anasetti, Claudio.
The New England Journal of Medicine
Dec 20, **2001**; 345 (25),pp 1794-1800
LINE COUNT: 00288 WORD COUNT: 03986

Abstract

Background: Successful engraftment of hematopoietic stem cells from unrelated donors is influenced by disparities between the donor and recipient for HLA-A, B, and C alleles. Disparities between HLA sequence polymorphisms that are serologically detectable are termed antigen mismatches, whereas those that can be identified only by DNA-based typing methods are termed allele mismatches. Whether both kinds of polymorphisms are important in transplantation is not known. We tested the hypothesis that allele mismatches that are detectable only at the DNA level are less immunogenic than those that are serologically detectable and thereby are associated with a lower risk of graft failure after hematopoietic-cell transplantation.

Methods: We used DNA sequencing to define the HLA-A, B, and C alleles in 471 patients who received **bone marrow** from unrelated donors for the treatment of chronic myeloid leukemia after myeloablative conditioning therapy. The odds ratios for graft failure were determined for recipients of transplants from donors with a single class I allele mismatch, a single class I antigen mismatch, or two or more class I mismatches, as compared with those with no mismatch.

Results: A single HLA allele mismatch did not increase the risk of graft failure, whereas a single antigen mismatch significantly increased the risk. The risk was also increased if the recipient was HLA homozygous at the mismatched class I locus or if the donor had two or more class I mismatches.

Conclusions: HLA class I antigen mismatches that are serologically detectable confer an enhanced risk of graft failure after hematopoietic-cell transplantation. Transplants from donors with a single class I allele mismatch that is not serologically detectable may be used without an increased risk of graft failure. (N Engl J Med 2001;345:1794-800.)

9/3,AB/107 (Item 26 from file: 444)
DIALOG(R)File 444:New England Journal of Med.

00122004

Fluconazole Prophylaxis against Fungal Colonization and Infection in Preterm Infants (Original Articles)

Kaufman, David; Boyle, Robert; Hazen, Kevin C.; Patrie, James T.;
Robinson, Melinda; Donowitz, Leigh Goodman.
The New England Journal of Medicine
Dec 6, **2001**; 345 (23),pp 1660-1666
LINE COUNT: 00387 WORD COUNT: 05342

Abstract

Background: Invasive fungal infection is associated with substantial morbidity and mortality in preterm infants. We evaluated the efficacy of prophylactic fluconazole in preventing fungal colonization and invasive infection in extremely-low-birth-weight infants.

Methods: We conducted a prospective, randomized, double-blind clinical trial over a 30-month period in 100 preterm infants with birth weights of less than 1000 g. The infants were randomly assigned during the first five days of life to receive either intravenous fluconazole or placebo for six weeks. We obtained weekly surveillance cultures from all patients.

Results: The 50 infants randomly assigned to fluconazole and the 50 control infants were similar in terms of birth weight, gestational age at birth, and base-line risk factors for fungal infection. During the six-week treatment period, fungal colonization was documented in 30 infants in the placebo group (60 percent) and 11 infants in the fluconazole group (22 percent; difference in risk, 0.38; 95 percent confidence interval, 0.18 to 0.56; P=0.002). Invasive fungal infection with positive growth of fungal isolates from the **blood**, urine, or cerebrospinal fluid developed in 10 infants in the placebo group (20 percent) and none of the infants in the fluconazole group (difference in risk, 0.20; 95 percent confidence interval, 0.04 to 0.36; P=0.008). The sensitivities of the fungal isolates to fluconazole did not change during the study, and no adverse effects of the fluconazole therapy were documented.

Conclusions: Prophylactic administration of fluconazole during the first six weeks of life is effective in preventing fungal colonization and invasive fungal infection in infants with birth weights of less than 1000 g. (N Engl J Med 2001;345:1660-6.)

9/3,AB/108 (Item 27 from file: 444)
DIALOG(R)File 444:New England Journal of Med.

00121989

Current Concepts: Mesenteric Venous Thrombosis (**Review** Article)

Kumar, Shaji; Sarr, Michael G.; Kamath, Patrick S.
The New England Journal of Medicine
Dec 6, **2001**; 345 (23),pp 1683-1688
LINE COUNT: 00310 WORD COUNT: 04283

9/3,AB/109 (Item 28 from file: 444)
DIALOG(R)File 444:New England Journal of Med.

00121969

Weekly Clinicopathological Exercises: Case 37-2001: A 76-Year-Old Man with Fever, Dyspnea, Pulmonary Infiltrates, Pleural Effusions, and Confusion (Case Records of the Massachusetts General Hospital)

Yawetz, Sigal; Mark, Eugene J.
The New England Journal of Medicine
Nov 29, **2001**; 345 (22),pp 1627-1634
LINE COUNT: 00441 WORD COUNT: 06098

9/3,AB/110 (Item 29 from file: 444)
DIALOG(R)File 444:New England Journal of Med.

00121949

Weekly Clinicopathological Exercises: Case 36-2001: Acute Febrile Respiratory Illness in a 57-Year-Old Man with Recurrent Pulmonary Disorders (Case Records of the Massachusetts General Hospital)

Dolin, Raphael; Mark, Eugene J.
The New England Journal of Medicine

Nov 22, **2001**; 345 (21),pp 1558-1565
LINE COUNT: 00339 WORD COUNT: 04685

9/3,AB/111 (Item 30 from file: 444)
DIALOG(R)File 444:New England Journal of Med.

00121922

Brief Report: Transmission of a T-Cell Lymphoma by Allogeneic **Bone Marrow** Transplantation (Original Articles)

Berg, Karin D.; Brinster, Nooshin K.; Huhn, Karen M.; Goggins, Michael G.; Jones, Richard J.; Makary, Adel; Murphy, Kathleen M.; Griffin, Constance A.; Rosenblum-Vos, Lynne S.; Borowitz, Michael J.; Nousari, Hossein C.; Eshleman, James R.
The New England Journal of Medicine
Nov 15, **2001**; 345 (20),pp 1458-1463
LINE COUNT: 00314 WORD COUNT: 04337

9/3,AB/112 (Item 31 from file: 444)
DIALOG(R)File 444:New England Journal of Med.

00121913

Weekly Clinicopathological Exercises: Case 34-2001: A 54-Year-Old Woman with Multiple Sclerosis, Prolonged Fever, and Skin Nodules (Case Records of the Massachusetts General Hospital)

Jacobson, Joseph O.; de Leval, Laurence.
The New England Journal of Medicine
Nov 8, **2001**; 345 (19),pp 1409-1415
LINE COUNT: 00364 WORD COUNT: 05030

9/3,AB/113 (Item 32 from file: 444)
DIALOG(R)File 444:New England Journal of Med.

00121869

Brief Report: BK-Related Polyomavirus Vasculopathy in a Renal-Transplant Recipient (Original Articles)

Petrogiannis-Haliotis, Tina; Sakoulas, George; Kirby, James; Koralnik, Igor J.; Dvorak, Ann M.; Monahan-Earley, Rita; De Girolami, Paola C.; De Girolami, Umberto; Upton, Melissa; Major, Eugene O.; Pfister, Luz-Andrea; Joseph, Jeffrey T.
The New England Journal of Medicine
Oct 25, **2001**; 345 (17),pp 1250-1255
LINE COUNT: 00355 WORD COUNT: 04902

9/3,AB/114 (Item 33 from file: 444)
DIALOG(R)File 444:New England Journal of Med.

00121826

Tuberculosis Associated with Infliximab, a Tumor Necrosis Factor alpha -Neutralizing Agent Original Articles

Keane, Joseph; Gershon, Sharon; Wise, Robert P.; Mirabile-Levens, Elizabeth; Kasznica, John; Schwieterman, William D.; Siegel, Jeffrey N.; Braun, M. Miles.
The New England Journal of Medicine
Oct 11, **2001**; 345 (15),pp 1098-1104
LINE COUNT: 00360 WORD COUNT: 04978

Abstract
Background: Infliximab is a humanized antibody against tumor necrosis factor (alpha) (TNF-(alpha)) that is used in the treatment of Crohn's disease and rheumatoid arthritis. Approximately 147,000 patients throughout the world have received infliximab. Excess TNF-(alpha) in association with tuberculosis may cause weight loss and night sweats, yet in animal models it has a protective role in the host response to tuberculosis. There is no direct evidence of a protective role of TNF-(alpha) in patients with tuberculosis.
Methods: We analyzed all reports of tuberculosis after infliximab therapy that had been received as of May 29, 2001, through the MedWatch spontaneous reporting system of the Food and Drug Administration.
Results: There were 70 reported cases of tuberculosis after treatment with infliximab for a median of 12 weeks. In 48 patients, tuberculosis developed after three or fewer infusions. Forty of the patients had extrapulmonary disease (17 had disseminated disease, 11 lymph-node disease, 4 peritoneal disease, 2 pleural disease, and 1 each meningeal, enteric, paravertebral, **bone**, genital, and bladder disease). The diagnosis was confirmed by a biopsy in 33 patients. Of the 70 reports, 64 were from countries with a low incidence of tuberculosis. The reported frequency of tuberculosis in association with infliximab therapy was much higher than the reported frequency of other opportunistic infections associated with this drug. In addition, the rate of reported cases of tuberculosis among patients treated with infliximab was higher than the available background rates.
Conclusions: Active tuberculosis may develop soon after the initiation of treatment with infliximab. Before prescribing the drug, physicians should screen patients for latent tuberculosis infection or disease. (N Engl J Med 2001;345:1098-104.)

9/3,AB/115 (Item 34 from file: 444)
DIALOG(R)File 444:New England Journal of Med.

00121741

Advances in Immunology: Immunomodulation of Autoimmune and Inflammatory Diseases with Intravenous Immune Globulin (**Review** Article)

Kazatchkine, Michel D.; Kaveri, Srini V.
The New England Journal of Medicine
Sep 6, **2001**; 345 (10),pp 747-755
LINE COUNT: 00433 WORD COUNT: 05982

9/3,AB/116 (Item 35 from file: 444)
DIALOG(R)File 444:New England Journal of Med.

00121625

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Advances in Immunology: Autoimmune Diseases **Review** Article

Davidson, Anne; Diamond, Betty.
The New England Journal of Medicine
Aug 2, **2001**; 345 (5),pp 340-350
LINE COUNT: 00532 WORD COUNT: 07354

9/3,AB/117 (Item 36 from file: 444)
DIALOG(R)File 444:New England Journal of Med.
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00121513
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Weekly Clinicopathological Exercises: Case 19-2001: A 50-Year-Old Man with Fever and Joint Pain (Case Records of the Massachusetts General Hospital)

Helfgott, Simon M.; Kratz, Alexander.
The New England Journal of Medicine
Jun 21, **2001**; 344 (25),pp 1929-1935
LINE COUNT: 00357 WORD COUNT: 04937

9/3,AB/118 (Item 37 from file: 444)
DIALOG(R)File 444:New England Journal of Med.
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00121392
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Controlling New Prion Diseases (Editorials)

Roos, Raymond P.
The New England Journal of Medicine
May 17, **2001**; 344 (20),pp 1548-1551
LINE COUNT: 00239 WORD COUNT: 03311

9/3,AB/119 (Item 38 from file: 444)
DIALOG(R)File 444:New England Journal of Med.
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00121261
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Drug Therapy: Interactions among Drugs for HIV and Opportunistic Infections (**Review** Articles)

Piscitelli, Stephen C.; Gallicano, Keith D.
The New England Journal of Medicine
Mar 29, **2001**; 344 (13),pp 984-996
LINE COUNT: 00580 WORD COUNT: 08006

9/3,AB/120 (Item 39 from file: 444)
DIALOG(R)File 444:New England Journal of Med.
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00121233
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Weekly Clinicopathological Exercises: Case 9-2001: A 64-Year-Old Woman with Peripheral Neuropathy, Paraproteinemia, and Lymphadenopathy (Case Records of the Massachusetts General Hospital)

Gorson, Kenneth C.; Hedley-Whyte, E. Tessa; Skinner, Martha M.
The New England Journal of Medicine
Mar 22, **2001**; 344 (12),pp 917-923
LINE COUNT: 00300 WORD COUNT: 04153

9/3,AB/121 (Item 40 from file: 444)
DIALOG(R)File 444:New England Journal of Med.

00121230

Use of Chemotherapy plus a Monoclonal Antibody against HER2 for Metastatic Breast Cancer That Overexpresses HER2 (Original Articles)

Slamon, Dennis J.; Leyland-Jones, Brian; Shak, Steven; Fuchs, Hank; Paton, Virginia; Bajamonde, Alex; Fleming, Thomas; Eiermann, Wolfgang; Wolter, Janet; Pegram, Mark; Baselga, Jose; Norton, Larry.
The New England Journal of Medicine
Mar 15, **2001**; 344 (11),pp 783-792
LINE COUNT: 00430 WORD COUNT: 05937

Abstract

Background: The HER2 gene, which encodes the growth factor receptor HER2, is amplified and HER2 is overexpressed in 25 to 30 percent of breast cancers, increasing the aggressiveness of the tumor.

Methods: We evaluated the efficacy and safety of trastuzumab, a recombinant monoclonal antibody against HER2, in women with metastatic breast cancer that overexpressed HER2. We randomly assigned 234 patients to receive standard chemotherapy alone and 235 patients to receive standard chemotherapy plus trastuzumab. Patients who had not previously received adjuvant (postoperative) therapy with an anthracycline were treated with doxorubicin (or epirubicin in the case of 36 women) and cyclophosphamide with (143 women) or without trastuzumab (138 women). Patients who had previously received adjuvant anthracycline were treated with paclitaxel alone (96 women) or paclitaxel with trastuzumab (92 women).

Results: The addition of trastuzumab to chemotherapy was associated with a longer time to disease progression (median, 7.4 vs. 4.6 months; P<0.001), a higher rate of objective response (50 percent vs. 32 percent, P<0.001), a longer duration of response (median, 9.1 vs. 6.1 months; P<0.001), a lower rate of death at 1 year (22 percent vs. 33 percent, P=0.008), longer survival (median survival, 25.1 vs. 20.3 months; P=0.046), and a 20 percent reduction in the risk of death. The most important adverse event was cardiac dysfunction, which occurred in 27 percent of the group given an anthracycline, cyclophosphamide, and trastuzumab; 8 percent of the group given an anthracycline and cyclophosphamide alone; 13 percent of the group given paclitaxel and trastuzumab; and 1 percent of the group given paclitaxel alone. Although the cardiotoxicity was potentially severe and, in some cases, life-threatening, the symptoms generally improved with standard medical management.

Conclusions: Trastuzumab increases the clinical benefit of first-line chemotherapy in metastatic breast cancer that overexpresses HER2. (N Engl J Med 2001;344:783-92.)

9/3,AB/122 (Item 41 from file: 444)
DIALOG(R)File 444:New England Journal of Med.

00121219
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Weekly Clinicopathological Exercises: Case 8-2001: A 61-Year-Old Man with Transient Quadriplegia and Apnea Case Records of the Massachusetts General Hospital

Wong, Eric T.; Louis, David N.
The New England Journal of Medicine
Mar 15, **2001**; 344 (11),pp 832-839
LINE COUNT: 00438 WORD COUNT: 06046

9/3,AB/123 (Item 42 from file: 444)
DIALOG(R)File 444:New England Journal of Med.

00121171
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Advances in Immunology: Tolerance and Autoimmunity (**Review** Articles)

Kamradt, Thomas; Mitchison, N. Avrion.
The New England Journal of Medicine
Mar 1, **2001**; 344 (9),pp 655-664
LINE COUNT: 00451 WORD COUNT: 06236

9/3,AB/124 (Item 43 from file: 444)
DIALOG(R)File 444:New England Journal of Med.

00121133
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Weekly Clinicopathological Exercises: Case 5-2001: A 52-Year-Old Man with Chronic Anemia and Sudden Severe Abdominal Pain (Case Records of the Massachusetts General Hospital)

Navab, Farhad; Yantiss, Rhonda K.
The New England Journal of Medicine
Feb 15, **2001**; 344 (7),pp 510-517
LINE COUNT: 00523 WORD COUNT: 07230

9/3,AB/125 (Item 44 from file: 444)
DIALOG(R)File 444:New England Journal of Med.

00121095
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Advances in Immunology: Asthma (**Review** Articles)

Busse, William W.; Lemanske, Robert F., Jr.
The New England Journal of Medicine
Feb 1, **2001**; 344 (5),pp 350-362
LINE COUNT: 00512 WORD COUNT: 07069

9/3,AB/126 (Item 45 from file: 444)
DIALOG(R)File 444:New England Journal of Med.

00121068

Weekly Clinicopathological Exercises: Case 3-2001: A 59-Year-Old Diabetic Man with Unilateral Visual Loss and Oculomotor-Nerve Palsy (Case Records of the Massachusetts General Hospital)

Brown, Richard B.; Lau, Stephen K..
The New England Journal of Medicine
Jan 25, **2001**; 344 (4),pp 286-293
LINE COUNT: 00476 WORD COUNT: 06571

9/3,AB/127 (Item 46 from file: 444)
DIALOG(R)File 444:New England Journal of Med.

00121062

Enzyme-Replacement Therapy in Mucopolysaccharidosis I (Original Articles)

Kakkis, Emil D.; Muenzer, Joseph; Tiller, George E.; Waber, Lewis; Belmont, John; Passage, Merry; Izykowski, Barbara; Phillips, Jeffrey; Doroshow, Robin; Walot, Irv; Hoft, Richard; Yu, Kian T.; Okazaki, Susie; Lewis, Dave; Thompson, Jerry N.; Neufeld, Elizabeth F.
The New England Journal of Medicine
Jan 18, **2001**; 344 (3),pp 182-188
LINE COUNT: 00401 WORD COUNT: 05547

Abstract
Background: Mucopolysaccharidosis I is a lysosomal storage disease caused by a deficiency of the enzyme (alpha)-L-iduronidase. We evaluated the effect of enzyme-replacement therapy with recombinant human (alpha)-L-iduronidase in patients with this disorder.
Methods: We treated 10 patients with mucopolysaccharidosis I (age, 5 to 22 years) with recombinant human (alpha)-L-iduronidase at a dose of 125,000 U per kilogram of body weight given intravenously once weekly for 52 weeks. The patients were evaluated at base line and at 6, 12, 26, and 52 weeks by detailed clinical examinations, magnetic resonance imaging of the abdomen and brain, echocardiography, range-of-motion measurements, polysomnography, clinical laboratory evaluations, measurements of leukocyte (alpha)-L-iduronidase activity, and urinary glycosaminoglycan excretion.
Results: Hepatosplenomegaly decreased significantly in all patients, and the size of the liver was normal for body weight and age in eight patients by 26 weeks. The rate of growth in height and weight had increased by a mean of 85 and 131 percent, respectively, at 52 weeks in the six prepubertal patients. The mean maximal range of motion of shoulder flexion and elbow extension increased significantly. The number of episodes of apnea and hypopnea during sleep decreased 61 percent. New York Heart Association functional class improved by one or two classes in all patients. Urinary glycosaminoglycan excretion decreased after three to four weeks of treatment; the mean reduction at 52 weeks was 63 percent of base-line values. Five patients had transient urticaria during infusions. Serum antibodies to (alpha)-L-iduronidase were detected in four patients.
Conclusions: In patients with mucopolysaccharidosis I, treatment with recombinant human (alpha)-L-iduronidase reduces lysosomal storage in the liver and ameliorates some clinical manifestations of the disease. (N Engl J Med 2001;344:182-8.)

9/3,AB/128 (Item 47 from file: 444)
DIALOG(R)File 444:New England Journal of Med.

00120915
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Advances in Immunology: Immunodeficiency Diseases Caused by Defects in Phagocytes **Review** Articles

Lekstrom-Himes, Julie A.; Gallin, John I.
The New England Journal of Medicine
Dec 7, **2000**; 343 (23),pp 1703-1714
LINE COUNT: 00506 WORD COUNT: 06986

9/3,AB/129 (Item 48 from file: 444)
DIALOG(R)File 444:New England Journal of Med.
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00120896
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Weekly Clinicopathological Exercises: Case 37-2000: An 11-Day-Old Boy with an Osteolytic Tibial Lesion (Case Records of the Massachusetts General Hospital)

Ready, John E.; Keel, Suzanne B.
The New England Journal of Medicine
Nov 30, **2000**; 343 (22),pp 1634-1638
LINE COUNT: 00282 WORD COUNT: 03899

9/3,AB/130 (Item 49 from file: 444)
DIALOG(R)File 444:New England Journal of Med.
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00120819
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Advances in Immunology: Primary Immunodeficiency Diseases Due to Defects in Lymphocytes (**Review** Article)

Buckley, Rebecca H.
The New England Journal of Medicine
Nov 2, **2000**; 343 (18),pp 1313-1324
LINE COUNT: 00570 WORD COUNT: 07874

9/3,AB/131 (Item 50 from file: 444)
DIALOG(R)File 444:New England Journal of Med.
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Weekly Clinicopathological Exercises: Case 33-2000: A Seven-Year-Old Girl with the Superior Vena Cava Syndrome after Treatment for a Peripheral Rhabdomyosarcoma (Case Records of the Massachusetts General Hospital)

Larsen, Eric; de Leval, Laurence.
The New England Journal of Medicine
Oct 26, **2000**; 343 (17),pp 1249-1257
LINE COUNT: 00507 WORD COUNT: 07001

9/3,AB/132 (Item 51 from file: 444)

00120576

Epstein-Barr Virus Infection **Review** Article

Cohen, Jeffrey I.
The New England Journal of Medicine
Aug 17, **2000**; 343 (7),pp 481-492
LINE COUNT: 00540 WORD COUNT: 07458

9/3,AB/133 (Item 52 from file: 444)

00120537

Hepatic Iron Concentration and Total Body Iron Stores in Thalassemia Major (Original Articles)

Angelucci, Emanuele; Brittenham, Gary M.; McLaren, Christine E.; Ripalti, Marta; Baronciani, Donatella; Giardini, Claudio; Galimberti, Maria; Polchi, Paola; Lucarelli, Guido.
The New England Journal of Medicine
Aug 3, **2000**; 343 (5),pp 327-331
LINE COUNT: 00314 WORD COUNT: 04339

Abstract
Background and Methods: We tested the usefulness of measuring the hepatic iron concentration to evaluate total body iron stores in patients who had been cured of thalassemia major by **bone marrow** transplantation and who were undergoing phlebotomy treatment to remove excess iron.
Results: We began treatment with phlebotomy a mean (+/-SD) of 4.3+/-2.7 years after transplantation in 48 patients without hepatic cirrhosis. In the group of 25 patients with liver-biopsy samples that were at least 1.0 mg in dry weight, there was a significant correlation between the decrease in the hepatic iron concentration and total body iron stores (r=0.98, P<0.001). Assuming that the hepatic iron concentration is reduced to zero with complete removal of body iron stores during phlebotomy, the amount of total body iron stores (in milligrams per kilogram of body weight) is equivalent to 10.6 times the hepatic iron concentration (in milligrams per gram of liver, dry weight). With the use of this equation, we could reliably estimate total body iron stores as high as 250 mg per kilogram of body weight, with a standard error of less than 7.9.
Conclusions: The hepatic iron concentration is a reliable indicator of total body iron stores in patients with thalassemia major. In patients with transfusion-related iron overload, repeated determinations of the hepatic iron concentration can provide a quantitative means of measuring the long-term iron balance. (N Engl J Med 2000;343:327-31.)

9/3,AB/134 (Item 53 from file: 444)

00120518

Weekly Clinicopathological Exercises: Case 23-2000: A 49-Year-Old Man with

the Acquired Immunodeficiency Syndrome and a Tibial Lesion Case Records of the Massachusetts General Hospital

Mayer, Kenneth H.; Rosenberg, Andrew E.
The New England Journal of Medicine
Jul 27, **2000**; 343 (4),pp 281-287
LINE COUNT: 00421 WORD COUNT: 05814

9/3,AB/135 (Item 54 from file: 444)
DIALOG(R)File 444:New England Journal of Med.

00120470

Advances in Immunology: The Immune System (Second of Two Parts) (**Review** Article)

Delves, Peter J.; Roitt, Ivan M.
The New England Journal of Medicine
Jul 13, **2000**; 343 (2),pp 108-117
LINE COUNT: 00536 WORD COUNT: 07406

9/3,AB/136 (Item 55 from file: 444)
DIALOG(R)File 444:New England Journal of Med.

00120448

Advances in Immunology: The Immune System (First of Two Parts) (**Review** Article)

Delves, Peter J.; Roitt, Ivan M.
The New England Journal of Medicine
Jul 6, **2000**; 343 (1),pp 37-49
LINE COUNT: 00712 WORD COUNT: 09832

9/3,AB/137 (Item 56 from file: 444)
DIALOG(R)File 444:New England Journal of Med.

00120419

Causes and Outcomes of the Acute Chest Syndrome in Sickle Cell Disease (Original Articles)

Vichinsky, Elliott P.; Neumayr, Lynne D.; Earles, Ann N.; Williams, Roger; Lennette, Evelyne T.; Dean, Deborah; Nickerson, Bruce; Orringer, Eugene; McKie, Virgil; Bellevue, Rita; Daeschner, Charles; Manci-Gardener, Elizabeth; Abboud, Miguel; Moncino, Mark; Ballas, Samir; Ware, Russell; for the National Acute Chest Syndrome Study Group.
The New England Journal of Medicine
Jun 22, **2000**; 342 (25),pp 1855-1865
LINE COUNT: 00425 WORD COUNT: 05865

Abstract
Background: The acute chest syndrome is the leading cause of death among patients with sickle cell disease. Since its cause is largely

unknown, therapy is supportive. Pilot studies with improved diagnostic techniques suggest that infection and fat embolism are underdiagnosed in patients with the syndrome.

Methods: In a 30-center study, we analyzed 671 episodes of the acute chest syndrome in 538 patients with sickle cell disease to determine the cause, outcome, and response to therapy. We evaluated a treatment protocol that included matched transfusions, bronchodilators, and bronchoscopy. Samples of **blood** and respiratory tract secretions were sent to central laboratories for antibody testing, culture, DNA testing, and histopathological analyses.

Results: Nearly half the patients were initially admitted for another reason, mainly pain. When the acute chest syndrome was diagnosed, patients had hypoxia, decreasing hemoglobin values, and progressive multilobar pneumonia. The mean length of hospitalization was 10.5 days. Thirteen percent of patients required mechanical ventilation, and 3 percent died. Patients who were 20 or more years of age had a more severe course than those who were younger. Neurologic events occurred in 11 percent of patients, among whom 46 percent had respiratory failure. Treatment with phenotypically matched transfusions improved oxygenation, with a 1 percent rate of alloimmunization. One fifth of the patients who were treated with bronchodilators had clinical improvement. Eighty-one percent of patients who required mechanical ventilation recovered. A specific cause of the acute chest syndrome was identified in 38 percent of all episodes and 70 percent of episodes with complete data. Among the specific causes were pulmonary fat embolism and 27 different infectious pathogens. Eighteen patients died, and the most common causes of death were pulmonary emboli and infectious bronchopneumonia. Infection was a contributing factor in 56 percent of the deaths.

Conclusions: Among patients with sickle cell disease, the acute chest syndrome is commonly precipitated by fat embolism and infection, especially community-acquired pneumonia. Among older patients and those with neurologic symptoms, the syndrome often progresses to respiratory failure. Treatment with transfusions and bronchodilators improves oxygenation, and with aggressive treatment, most patients who have respiratory failure recover. (N Engl J Med 2000;342:1855-65.)

9/3,AB/138 (Item 57 from file: 444)
DIALOG(R)File 444:New England Journal of Med.

00120360

Medical Progress: Pediatric Surgery (Second of Two Parts) (**Review** Articles)

Adzick, N. Scott; Nance, Michael L.
The New England Journal of Medicine
Jun 8, **2000**; 342 (23),pp 1726-1732
LINE COUNT: 00457 WORD COUNT: 06311

9/3,AB/139 (Item 58 from file: 444)
DIALOG(R)File 444:New England Journal of Med.

00120335

Brief Report: Transfusions of Polymerized Bovine Hemoglobin in a Patient with Severe Autoimmune Hemolytic Anemia (Original Articles)

Mullon, John; Giacoppe, George; Clagett, Cynthia; McCune, David;

Dillard, Thomas.
The New England Journal of Medicine
Jun 1, **2000**; 342 (22),pp 1638-1643
LINE COUNT: 00304   WORD COUNT: 04206

9/3,AB/140   (Item 59 from file: 444)
DIALOG(R)File 444:New England Journal of Med.

00120291

Weekly Clinicopathological Exercises: Case 15-2000: A 69-Year-Old Man with Myasthenia Gravis and a Mediastinal Mass (Case Records of the Massachusetts General Hospital)

Siao, Peter; Zukerberg, Lawrence R.
The New England Journal of Medicine
May 18, **2000**; 342 (20),pp 1508-1514
LINE COUNT: 00514   WORD COUNT: 07100

9/3,AB/141   (Item 60 from file: 444)
DIALOG(R)File 444:New England Journal of Med.

00120242

Testing for Polyomavirus Type BK DNA in Plasma to Identify Renal-Allograft Recipients with Viral Nephropathy (Original Articles)

Nickeleit, Volker; Klimkait, Thomas; Binet, Isabelle F.; Dalquen, Peter; Del Zenero, Veronika; Thiel, Gilbert; Mihatsch, Michael J.; Hirsch, Hans H.
The New England Journal of Medicine
May 4, **2000**; 342 (18),pp 1309-1315
LINE COUNT: 00402   WORD COUNT: 05551

Abstract
Background: Reactivation of polyomavirus type BK (BK virus) is increasingly recognized as a cause of severe renal-allograft dysfunction. Currently, patients at risk for nephropathy due to infection with the BK virus are identified by the presence of cells containing viral inclusion bodies (``decoy cells'') in the urine or by biopsy of allograft **tissue**.
Methods: In a retrospective analysis, we performed polymerase-chain-reaction assays for BK virus DNA in plasma samples from 9 renal-allograft recipients with BK virus nephropathy; 41 renal-allograft recipients who did not have signs of nephropathy, 16 of whom had decoy cells in the urine; and as immunocompromised controls, 17 patients who had human immunodeficiency virus type 1 (HIV-1) infection (stage C3 according to the classification of the Centers for Disease Control and Prevention) and who had not undergone transplantation.
Results: In all nine patients with BK virus nephropathy, BK virus DNA was detected in the plasma at the time of the initial histologic diagnosis (a mean +/-SD] of 46+/-28 weeks after transplantation) and during the course of histologically diagnosed, persistent BK virus disease. In three of the six patients with nephropathy who were studied serially after transplantation, BK virus DNA was initially undetectable but was detected 16 to 33 weeks before nephropathy became clinically evident and was confirmed by biopsy. Tests for BK virus DNA in plasma became negative and the nephropathy resolved after the doses of immunosuppressive drugs were

decreased in two patients and after removal of the renal allograft in three patients. BK virus DNA was found in the plasma of only 2 of the 41 renal allograft recipients who had no signs of nephropathy and in none of the patients with HIV-1 infection.

Conclusions: Testing for BK virus DNA in plasma from renal-allograft recipients with use of the polymerase chain reaction is a sensitive and specific method for identifying viral nephropathy. N Engl J Med 2000;342:1309-15.

9/3,AB/142 (Item 61 from file: 444)
DIALOG(R)File 444:New England Journal of Med.

00120224

Medical Progress: Myelofibrosis with Myeloid Metaplasia (**Review** Articles)

Tefferi, Ayalew.
The New England Journal of Medicine
Apr 27, **2000**; 342 (17),pp 1255-1265
LINE COUNT: 00500 WORD COUNT: 06910

9/3,AB/143 (Item 62 from file: 444)
DIALOG(R)File 444:New England Journal of Med.

00120049

Weekly Clinicopathological Exercises: Case 5-2000: A 35-Year-Old Man with a Painful Abdominal Mass and Fever (Case Records of the Massachusetts General Hospital)

Daily, Johanna P.; Sadeghi, Saha.
The New England Journal of Medicine
Feb 17, **2000**; 342 (7),pp 493-500
LINE COUNT: 00525 WORD COUNT: 07249

9/3,AB/144 (Item 63 from file: 444)
DIALOG(R)File 444:New England Journal of Med.

00119979

Weekly Clinicopathological Exercises: Case 3-2000: A 66-Year-Old Woman with Diabetes, Coronary Disease, Orthostatic Hypotension, and the Nephrotic Syndrome (Case Records of the Massachusetts General Hospital)

DiSalvo, Thomas G.; King, Mary Etta; Smith, R. Neal.
The New England Journal of Medicine
Jan 27, **2000**; 342 (4),pp 264-273
LINE COUNT: 00548 WORD COUNT: 07566

9/3,AB/145 (Item 64 from file: 444)
DIALOG(R)File 444:New England Journal of Med.

00119952

Prediction of Adverse Outcomes in Children with Sickle Cell Disease
Original Articles

Miller, Scott T.; Sleeper, Lynn A.; Pegelow, Charles H.; Enos, Laura E.; Wang, Winfred C.; Weiner, Steven J.; Wethers, Doris L.; Smith, Jeanne; Kinney, Thomas R.
The New England Journal of Medicine
Jan 13, **2000**; 342 (2),pp 83-89
LINE COUNT: 00436 WORD COUNT: 06019

Abstract

Background: The ability to identify infants with sickle cell anemia who are likely to have severe complications later in life would permit accurate prognostication and tailoring of therapy to match disease-related risks and facilitate planning of clinical trials. We attempted to define the features of such babies by following the clinical course of 392 children with sickle cell disease from infancy to about the age of 10 years.

Methods: We analyzed the records of 392 infants who received the diagnosis of homozygous sickle cell anemia or sickle cell-(beta)(sup 0)-thalassemia before the age of six months and for whom comprehensive clinical and laboratory data were recorded prospectively; data were available for a mean (+/-SD) of 10.0+/-4.8 years. Results obtained before the age of two years were evaluated to determine whether they predicted the outcome later in life.

Results: Of the 392 infants in the cohort, 70 (18 percent) subsequently had an adverse outcome, defined as death (18 patients 26 percent]), stroke (25 36 percent]), frequent pain (17 24 percent]), or recurrent acute chest syndrome (10 14 percent]). Using multivariate analysis, we found three statistically significant predictors of an adverse outcome: an episode of dactylitis (defined as pain and tenderness in the hands or feet) before the age of one year (relative risk of an adverse outcome, 2.55; 95 percent confidence interval, 1.39 to 4.67), a hemoglobin level of less than 7 g per deciliter (relative risk, 2.47; 95 percent confidence interval, 1.14 to 5.33), and leukocytosis in the absence of infection (relative risk, 1.80; 95 percent confidence interval, 1.05 to 3.09).

Conclusions: Three easily identifiable manifestations of sickle cell disease that may appear in the first two years of life (dactylitis, severe anemia, and leukocytosis) can help to predict the possibility of severe sickle cell disease later in life. (N Engl J Med 2000;342:83-9.)

9/3,AB/146 (Item 65 from file: 444)
DIALOG(R)File 444:New England Journal of Med.

00119937

Impatient Inpatient Care (Clinical Problem Solving)

Gulati, Mridu; Saint, Sanjay; Tierney, Lawrence M., Jr.
The New England Journal of Medicine
Jan 6, **2000**; 342 (1),pp 37-40

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13mar03 11:31:42 User242957 Session D611.2
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File 5:Biosis Previews(R) 1969-2003 Feb W4
(c) 2003 BIOSIS
*File 5: Alert feature enhanced for multiple files, duplicates removal, customized scheduling. See HELP ALERT.

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Set	Items	Description
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S1	2141	SPARC OR OSTEONECTIN

? s s1 and py<1998 and (antisens? or ribozym?)
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Set	Items	Description
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	20950108	PY<1998
	37175	ANTISENS?
	6403	RIBOZYM?
S2	9	S1 AND PY<1998 AND (ANTISENS? OR RIBOZYM?)

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? t s3/3,ab/all

3/3,AB/1 (Item 1 from file: 155)
DIALOG(R)File 155:MEDLINE(R)

09272087 97170962 PMID: 9018235
Suppression of **SPARC** expression by **antisense** RNA abrogates the tumorigenicity of human melanoma cells.
Ledda M F; Adris S; Bravo A I; Kairiyama C; Bover L; Chernajovsky Y; Mordoh J; Podhajcer O L
Instituto de Investigaciones Bioquimicas, Fundacion Campomar, Buenos Aires, Argentina.
Nature medicine (UNITED STATES) Feb **1997**, 3 (2) p171-6, ISSN 1078-8956 Journal Code: 9502015
Document type: Journal Article
Languages: ENGLISH
Main Citation Owner: NLM
Record type: Completed
Acquisition of invasive/metastatic potential is a key event in tumor progression. Cell surface glycoproteins and their respective matrix ligands have been implicated in this process. Recent evidence reveals that the secreted glycoprotein **SPARC** (secreted protein, acidic and rich in cysteine) is highly expressed in different malignant tissues. The present study reports that the suppression of **SPARC** expression by human melanoma cells using a **SPARC antisense** expression vector results in a significant decrease in the in vitro adhesive and invasive capacities of tumor cells, completely abolishing their in vivo tumorigenicity. This is the first evidence that **SPARC** plays a key role in human melanoma invasive metastatic phenotype development.

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            1484  OSTEONECTIN?
           32795  ANTISENS?
            5711  RIBOZYM?
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2/3,AB/1 (Item 1 from file: 155)
DIALOG(R)File 155:MEDLINE(R)

09257900 97170962 PMID: 9018235

Suppression of SPARC expression by **antisense** RNA abrogates the tumorigenicity of human melanoma cells.

Ledda MF; Adris S; Bravo AI; Kairiyama C; Bover L; Chernajovsky Y; Mordoh J; Podhajcer OL

Instituto de Investigaciones Bioquimicas, Fundacion Campomar, Buenos Aires, Argentina.

Nature medicine (UNITED STATES) Feb 1997, 3 (2) p171-6, ISSN 1078-8956 Journal Code: CG5

Languages: ENGLISH

Document type: Journal Article

Record type: Completed

Acquisition of invasive/metastatic potential is a key event in tumor progression. Cell surface glycoproteins and their respective matrix ligands have been implicated in this process. Recent evidence reveals that the secreted glycoprotein SPARC (secreted protein, acidic and rich in cysteine) is highly expressed in different malignant tissues. The present study reports that the suppression of SPARC expression by human melanoma cells using a SPARC **antisense** expression vector results in a significant decrease in the in vitro adhesive and invasive capacities of tumor cells, completely abolishing their in vivo tumorigenicity. This is the first evidence that SPARC plays a key role in human melanoma invasive-metastatic phenotype development.

2/3,AB/2 (Item 2 from file: 155)
DIALOG(R)File 155:MEDLINE(R)

09049533 96341098 PMID: 8734932

[The role of SPARC gene in tumorigenic capacity of human melanoma cells]

Rol del gen SPARC en la capacidad tumorigenica de celulas de melanoma humano.

Ledda MF; Adris S; Bover L; Bravo AL; Mordoh J; Podhajcer OL

Instituto de Investigaciones Bioquimicas Luis Leloir, Fundacion Campoma, Buenos Aires, Argentina.

Medicina (ARGENTINA) 1996, 56 (1) p51-4, ISSN 0025-7680
Journal Code: MMM

Languages: SPANISH

Document type: Journal Article

Record type: Completed

Previous studies from our laboratory have demonstrated that human melanoma cell lines and tumors expressed high levels of the extracellular protein SPARC. In order to demonstrate its role in human melanoma progression, IIB-MEL-LES human melanoma cells were transfected with SPARC full length c-DNA in the **antisense** orientation. In vivo studies demonstrated that all the control mice injected with parental cells developed tumors, while none of the mice injected with cells obtained from three different clones with diminished levels of SPARC expression, developed tumors. These studies suggest that SPARC may play a key role in human melanoma progression.

2/3,AB/3 (Item 3 from file: 155)
DIALOG(R)File 155:MEDLINE(R)

09017425 96361504 PMID: 8717113
The impact of **osteonectin** for differential diagnosis of osteogenic bone tumors: an immunohistochemical and in situ hybridization approach.
Park YK; Yang MH; Park HR
Department of Pathology, College of Medicine, Kyung Hee University Medical Center, Seoul, Korea.
Skeletal radiology (GERMANY) Jan 1996, 25 (1) p13-7, ISSN 0364-2348 Journal Code: UTE
Languages: ENGLISH
Document type: Journal Article
Record type: Completed
Thirty-three osteosarcomas at various grades of histologic differentiation, including chondroblastic, osteoblastic, and fibroblastic variants, were investigated immunohistochemically for evidence of **osteonectin** . Twenty-two cases of varying types of osteosarcoma were examined with in situ hybridization for mRNA expression of **osteonectin**. Immunohistochemically, **osteonectin** was present in all the osteosarcomas in this study. With in situ hybridization, 12 out of 22 osteosarcomas showed a positive signal. Two osteochondrosarcomas, seven chondrosarcomas, and one mesenchymal chondrosarcoma were also studied with regard to the localization of **osteonectin** , either immunohistochemically or by in situ hybridization. Immunohistochemically, **osteonectin** was present in all the chondroid lesions except for one osteochondroma. However, in situ hybridization of **osteonectin** mRNA was negative in all the chondroid lesions we studied. This study revealed that immunohistochemical localization of **osteonectin** is not useful in providing conclusive diagnosis of osteosarcoma. In situ hybridization of **osteonectin** mRNA might be useful in differentiating osteosarcoma from nonsteogenic bone tumors.

2/3,AB/4 (Item 4 from file: 155)
DIALOG(R)File 155:MEDLINE(R)

08798440 96017858 PMID: 7556570
Extracellular matrix components in intestinal development.
Simon-Assmann P; Kedinger M; De Arcangelis A; Rousseau V; Simo P
INSERM U.381, Strasbourg, France.
Experientia (SWITZERLAND) Sep 29 1995, 51 (9-10) p883-900, ISSN 0014-4754 Journal Code: EQZ
Languages: ENGLISH
Document type: Journal Article; Review; Review, Academic
Record type: Completed
Intestinal morphogenesis and differentiation are dependent on heterotypic cell interactions between embryonic epithelial cells (endoderm) and stromal cells (mesenchyme). Extracellular matrix molecules represent attractive candidates for regulators of these interactions. The structural and functional diversity of the extracellular matrix as intestinal development proceeds is demonstrated by 1) spatio-temporal specific expression of the classically described constituents, 2) the finding of laminin and collagen IV variants, 3) changes in the ratio of individual constituent chains, and 4) a stage-specific regulation of basement membrane molecule production, in particular by glucocorticoids. The orientation/assembly of these extracellular matrix molecules could direct precise cellular functions through interactions via integrin molecules. The involvement of extracellular matrix, and in particular basement membrane molecules in heterotypic cell interactions leading to epithelial cell differentiation, has been highlighted by the use of experimental models such as cocultures, hybrid intestines and **antisense** approaches. These models allowed us to conclude that a correct elaboration and assembly of the basement

Osteonectin in matrix remodeling. A plasminogen-**osteonectin** -collagen complex.

Kelm RJ; Swords NA; Orfeo T; Mann KG
University of Vermont, Department of Biochemistry, College of Medicine, Burlington 05405.

Journal of biological chemistry (UNITED STATES) Dec 2 **1994**, 269 (48) p30147-53, ISSN 0021-9258 Journal Code: HIV
Contract/Grant No.: P01-AG 08777, AG, NIA
Languages: ENGLISH
Document type: Journal Article
Record type: Completed

Osteonectin is an adhesive glycoprotein synthesized constitutively by osteoblasts, endothelial cells, and megakaryocytes. Bone-derived and platelet-derived **osteonectins** differ in their electrophoretic mobility and carbohydrate content, and each displays different affinities for collagen matrices. Both types of **osteonectin** bind to plasminogen (Kd(app), of 4.7 +/- 1.0 x 10(-8) M for bone **osteonectin** and 1.2 +/- 0.1 x 10(-7) M for platelet **osteonectin**). The **osteonectin** -plasminogen interaction is **inhibited** by alpha 2-antiplasmin and epsilon-aminocaproic acid, suggesting that the interaction is mediated through the kringle 1 region of plasminogen. Both **osteonectins** enhance the rate of plasmin generation by tissue-type plasminogen activator to approximately the same extent as fibrinogen. Equilibrium binding measurements conducted using total internal reflection fluorescence spectroscopy indicate that plasminogen binds to collagen in the presence of bone **osteonectin** (Kd = 1.30 +/- 0.1 x 10(-7) M). No binding of plasminogen to collagen matrix was detected in the presence of platelet **osteonectin** or in the absence of bone **osteonectin**. Bone **osteonectin** -dependent binding of plasminogen to collagen matrix is reversed by the addition of epsilon-aminocaproic acid. The ability of both types of **osteonectin** to bind to and influence plasminogen activation and of bone **osteonectin** to anchor plasminogen on collagen matrices suggests that **osteonectin** may play a role in directing extracellular matrix proteolysis.

6/3,AB/6 (Item 6 from file: 155)
DIALOG(R)File 155:MEDLINE(R)

08422384 95105262 PMID: 7528753

Differential effects of retinoic acid and growth factors on osteoblastic markers and CD10/NEP activity in stromal-derived osteoblasts.

Benayahu D; Fried A; Shamay A; Cunningham N; Blumberg S; Wientroub S
Department of Histology and Cell Biology, Sackler Faculty of Medicine, Tel Aviv University, Israel.

Journal of cellular biochemistry (UNITED STATES) Sep **1994**, 56 (1) p62-73, ISSN 0730-2312 Journal Code: HNF
Languages: ENGLISH
Document type: Journal Article
Record type: Completed

The effects of retinoic acid (RA) on the expression of osteoblastic-related cell markers was examined. A marrow stromal osteogenic cell line, MBA-15, was analyzed by Northern blotting for the expression of bone matrix proteins. These cells constitutively express mRNA encoding for procollagen alpha 2 (I), **osteonectin** , osteopontin, biglycan, and alkaline phosphatase (ALK-P). Gene expression was unchanged in response to RA triggering for 24 hr. Furthermore, cell growth and enzymatic activities of ALK-P and neutral endopeptidase (CD10/NEP) were studied. These parameters were examined in MBA-15 and clonal populations representing different stages of differentiation. The cell's growth rate was unchanged, while ALK-P activity was greatly increased during the culture period under RA treatment in MBA-15 and in the clonal cell lines examined while CD10/NEP activity displayed a different pattern. MBA-15.4, a preosteoblast cell line, exhibited an **inhibition** in CD10/NEP activity at the beginning

of the culture period, reaching basal level with time. This activity was greatly increased over control level in MBA-15.6, a mature stage of osteoblasts. Furthermore, the response of cell lines to various growth factors was tested subsequent to priming the cultures with RA. A synergistic effect was monitored for ALK-P activity in MBA-15.4 and MBA-15.6 cells under rh-bone morphogenic protein (BMP-2) and purified osteogenin (BMP-3), and an antagonist effect was measured when cells were exposed to transforming growth factor beta (TGF beta). Contrarily, BMP-2 and BMP-3 **inhibited** the CD10/NEP activity that had remained unchanged following priming of the cell with RA. Insulin-like growth factor I (IGF-I) and basic fibroblast growth factors (bFGF) did not affect either ALK-P nor CD10/NEP activities in both cloned cells. Cellular response to bone-seeking hormone, parathyroid hormone (PTH), and prostaglandin E2 (PGE2) was monitored by activation of intracellular cAMP. Treatment with RA caused a dramatic decrease in MBA-15.6 cell responses to PTH and PGE2, but no significant effects could be observed in other clonal lines.

6/3,AB/7 (Item 7 from file: 155)
DIALOG(R)File 155:MEDLINE(R)

08382016 95034487 PMID: 7524643
Effect of a derivatized dextran on **human** osteoblast growth and phenotype expression.
Berrada S; Amedee J; Avramoglou T; Jozefonvicz J; Harmand MF
INSERM-U, 306, Universite de Bordeaux II, France.
Journal of biomaterials science. Polymer edition (NETHERLANDS) **1994**, 6 (2) p211-22, ISSN 0920-5063 Journal Code: AY7
Languages: ENGLISH
Document type: Journal Article
Record type: Completed
Water soluble derivatized dextran named E9 with a molecular weight of 45,000 g l-1 containing 58% methyl carboxylic acid unit, 19% benzylamide unit, and 26% sulfonate with a specific anticoagulant activity of 0.29 IU mg-1 was studied for its effects on **human** osteoblast growth and phenotype expression for short-term treatment. At concentrations between 1 ng ml-1 and 1 microgram ml-1 E9 has no effect on DNA synthesis whereas at higher concentrations DNA synthesis is **inhibited** in a dose related fashion (87% for 400 micrograms ml-1). For concentrations which do not modify osteoblast growth, E9 promotes alkaline phosphatase activity, type I collagen and osteocalcin synthesis with a maximum effect for 0.1-1 microgram ml-1. It has a synergistic effect with hPTH increasing AMPc. Moreover, **osteonectin** synthesis was enhanced in a dose-dependent manner between 0.1 and 5 micrograms ml-1. These results seem to indicate that E9 is able to stimulate **human** osteoblast phenotype expression and could be useful in clinical applications.

6/3,AB/8 (Item 8 from file: 155)
DIALOG(R)File 155:MEDLINE(R)

08348860 95169232 PMID: 7865094
Gingival crevicular fluid: biomarkers of periodontal tissue activity.
Embery G; Waddington R
Department of Basic Dental Science, Dental School, University of Wales College of Medicine, Cardiff, UK.
Advances in dental research (UNITED STATES) Jul **1994**, 8 (2) p329-36, ISSN 0895-9374 Journal Code: ADD
Languages: ENGLISH
Document type: Journal Article; Review; Review, Tutorial
Record type: Completed
The lack of precise clinical criteria for assessment of periodontal disease has led to a search for alternative means of determining active disease sites, prognosis of future sites of breakdown, and response to

therapy. This review highlights the potential array of biomarkers present in gingival crevicular fluid and which may relate to existing or predicted tissue regions undergoing metabolic change and derived from bacterial or host-cell-derived products. Among the former may be listed endotoxin, amines, butyrate, and a variety of enzymes and their **inhibitors**, such as trypsin-like proteases and bacterial collagenase. Arising from host cells is a variety of leucocytic hydrolase enzymes, lactoferrin, and lysozyme. These appear to be useful inflammatory markers and may be distinguished from products of connective tissue breakdown which include collagenous and non-collagenous products, including collagen peptides, **osteonectin**, and fibronectin. The proteoglycans have found particular favor as biomarkers of possible bone-resorptive activity. Attention has also been directed at the immune response, including comment on immunoglobulins, complement, eicosanoids, and cytokines. This review lists available information on the presence of these in gingival sulcus fluid and wherever possible relates their presence to disease activity.

6/3,AB/9 (Item 9 from file: 155)
DIALOG(R)File 155:MEDLINE(R)

08119065 94164428 PMID: 8119487
The biology of SPARC, a protein that modulates cell-matrix interactions.
Lane TF; Sage EH
Department of Genetics, Howard Hughes Medical Institute, Harvard University School of Medicine, Boston, Massachusetts 02115.
FASEB journal (UNITED STATES) Feb **1994**, 8 (2) p163-73, ISSN 0892-6638 Journal Code: FAS
Contract/Grant No.: GM40711, GM, NIGMS; HL18645, HL, NHLBI
Languages: ENGLISH
Document type: Journal Article; Review; Review, Tutorial
Record type: Completed
An extracellular matrix-associated glycoprotein expressed in a variety of tissues during embryogenesis and repair, SPARC contains modular domains that can function independently to bind cells and matrix components. Because SPARC can selectively disrupt cellular contacts with matrix and thereby effect changes in cell shape, it has been referred to as an antiadhesin. **Inhibition** of the expression of SPARC altered axial development in frogs, and deregulated expression in nematode worms resulted in a derangement of muscle attachment and embryonic lethality. SPARC also **inhibits** cell cycle progression in vitro, in part through a cationic, disulfide-bonded region that is homologous to a repeated domain in the cytokine **inhibitor** , follistatin. Moreover, SPARC binds specifically to the B chain of platelet-derived growth factor and alters the response of cells to several cytokines. Although information concerning the expression, biochemical properties, and cellular activities of SPARC has increased significantly over the last decade, the precise function of the protein has not been resolved. Goals of future studies include characterization of cell-surface receptors for SPARC and the interactions with morphogens and growth factors that regulate specific activities during animal development.

6/3,AB/10 (Item 10 from file: 155)
DIALOG(R)File 155:MEDLINE(R)

08105404 94102200 PMID: 8275945
Differentiation of **human** bone marrow osteogenic stromal cells in vitro: induction of the osteoblast phenotype by dexamethasone.
Cheng SL; Yang JW; Rifas L; Zhang SF; Avioli LV
Division of Bone and Mineral Diseases, Washington University School of Medicine, St. Louis, Missouri.
Endocrinology (UNITED STATES) Jan **1994**, 134 (1) p277-86, ISSN 0013-7227 Journal Code: EGZ
Contract/Grant No.: 5P01-AR-32087-09, AR, NIAMS

Languages: ENGLISH
Document type: Journal Article
Record type: Completed

Human bone marrow stromal cells were examined for their osteogenic potential in an in vitro cell culture system. Dexamethasone (Dex) treatment induced morphological transformation of these cells from an elongated to a more cuboidal shape, increased their alkaline phosphatase activity and cAMP responses to PTH and prostaglandin E2, and was essential for mineralization of the extracellular matrix. Dex-induced differentiation of **human** bone marrow stromal cells was apparent after 2-3 days of treatment and reached a maximum at 7-14 days, as judged by alkaline phosphatase activity, although induction of osteocalcin by 1,25-dihydroxyvitamin D3 was attenuated by Dex. Withdrawal of Dex resulted in an enhancement of the 1,25-dihydroxyvitamin D3-induced secretion of osteocalcin, whereas alkaline phosphatase activity and the cAMP response to PTH remained at prewithdrawal levels. The steady state mRNA level of **osteonectin** was not affected by Dex. Our results, which demonstrate that Dex conditions the differentiation of **human** bone marrow osteogenic stromal cells into osteoblast-like cells, support the hypothesis of a permissive effect of glucocorticoids in ensuring an adequate supply of mature osteoblast populations. Furthermore, the established **human** bone marrow stromal cell culture provides a good model of an in vitro system to study the regulation of differentiation of **human** bone osteoprogenitor cells.

6/3,AB/11 (Item 11 from file: 155)
DIALOG(R)File 155:MEDLINE(R)

08081116 94062822 PMID: 7916692

Molecular analysis of chicken embryo SPARC (**osteonectin**).

Bassuk JA; Iruela-Arispe ML; Lane TF; Benson JM; Berg RA; Sage EH
Department of Biological Structure, University of Washington, Seattle 98195.

European journal of biochemistry (GERMANY) Nov 15 **1993**, 218 (1) p117-27, ISSN 0014-2956 Journal Code: EMZ
Contract/Grant No.: AG-00057, AG, NIA; AR-31839, AR, NIAMS; GM-40711, GM, NIGMS

Languages: ENGLISH
Document type: Journal Article
Record type: Completed

SPARC is a secreted glycoprotein that modulates cell shape and cell-matrix interactions. Levels of SPARC are increased at sites of somitogenesis, osteogenesis, and angiogenesis in the embryo and during wound repair in the adult. We have cloned and characterized SPARC from chicken embryo. A 2.2-kbp cDNA, obtained by a novel use of the polymerase chain reaction, was determined to encode a 298-residue protein that is 85% identical to **human** SPARC. Antigenic sites in particular appear to be highly conserved, as antibodies against C-terminal sequences of murine and bovine SPARC reacted with a 41-43 kDa protein in chicken embryo extracts. Chicken SPARC can be defined by four sequence signatures: (a) a conserved spacing of 11 cysteine residues in domain II, (b) the pentapeptide KKGHK in domain II, which is contained within a larger region of 31 identical residues, (c) a 100% conserved region of 10 residues in domain III, and (d) a C-terminal, calcium-binding EF-hand motif. SPARC mRNAs in the 10-day-old chicken embryo are represented by three sizes of 1.8, 2.2 and 3.0 kb. The relative steady-state levels for the 2.2-kb mRNA were determined as aorta > or = skeletal muscle > calvarium > vertebra > anterior limb > kidney > heart > brain > skin and lung >> liver. The relative abundance of the 1.8-kb and 2.2-kb mRNAs varied among tissues and indicated that differential processing of SPARC mRNAs might occur. All three RNA species were detected by a cDNA probe for the N-terminal part of the coding region. Thus, the three mRNA species appear to arise from differential 3' splicing and/or polyadenylation. Collective evidence demonstrates that SPARC has been well-conserved during vertebrate evolution, a finding that indicates a

fundamental role for this protein in development.

6/3,AB/12 (Item 12 from file: 155)
DIALOG(R)File 155:MEDLINE(R)

08069201 93123351 PMID: 8419407
Differential effects of SPARC and cationic SPARC peptides on DNA synthesis by endothelial cells and fibroblasts.
Funk SE; Sage EH
Department of Biological Structure, School of Medicine, University of Washington, Seattle 98195.
Journal of cellular physiology (UNITED STATES) Jan **1993**, 154 (1) p53-63, ISSN 0021-9541 Journal Code: HNB
Contract/Grant No.: GM 40711, GM, NIGMS; HL 18645, HL, NHLBI
Languages: ENGLISH
Document type: Journal Article
Record type: Completed
SPARC (secreted protein, acidic and rich in cysteine), also known as **osteonectin**, is an extracellular Ca+2-binding glycoprotein that **inhibits** the incorporation of [3H]-thymidine and delays the onset of S-phase in synchronized cultures of bovine aortic endothelial (BAE) cells. This effect appears not to be dependent on the functional properties of SPARC associated with changes in cell shape or **inhibition** of cell spreading. In this study we investigate the conditions under which cell cycle modulation occurs in different types of cells. **Human** umbilical vein endothelial cells, a transformed fetal BAE cell line, and bovine capillary endothelial cells exhibited a sensitivity to SPARC and a cationic peptide from a non-Ca+2-binding region of SPARC (peptide 2.1, 0.2-0.8 mM) similar to that observed in BAE cells. In contrast, **human** foreskin fibroblasts and fetal bovine ligament fibroblasts exhibited an increase in the incorporation of [3H]-thymidine in the presence of 25 microM-0.2 mM peptide 2.1; **inhibition** was observed at concentrations in excess of 0.4 mM. This biphasic modulation could be further localized to a sequence of 10 amino acids comprising the N-terminal half of peptide 2.1. A synthetic peptide from another cationic region of SPARC (peptide 2.3) increased [3H]-thymidine incorporation by BAE cells and fibroblasts in a dose-dependent manner. In endothelial cells, a stimulation of 50% was observed at a concentration of 0.01 mM; fibroblasts required approximately 100-fold more peptide 2.3 for levels of stimulation comparable to those obtained in endothelial cells. The observation that SPARC and unique SPARC peptides can differentially influence the growth of fibroblasts and endothelial cells in a concentration-dependent manner suggests that SPARC might regulate proliferation of specific cells during wound repair and remodeling.

6/3,AB/13 (Item 13 from file: 155)
DIALOG(R)File 155:MEDLINE(R)

08036381 93272857 PMID: 8500547
Modulation of SPARC expression during butyrate-induced terminal differentiation of cultured **human** keratinocytes: regulation via a TGF-beta-dependent pathway.
Ford R; Wang G; Jannati P; Adler D; Racanelli P; Higgins PJ; Staiano-Coico L
Department of Surgery, Cornell University Medical College, New York, New York 10021.
Experimental cell research (UNITED STATES) Jun **1993**, 206 (2) p261-75, ISSN 0014-4827 Journal Code: EPB
Contract/Grant No.: GM26145, GM, NIGMS; GM42461, GM, NIGMS
Languages: ENGLISH
Document type: Journal Article
Record type: Completed

Expression of SPARC (secreted protein acidic and rich in cysteine), a 43-kDa extracellular matrix-associated glycoprotein involved in tissue remodeling, was quantitated during normal **human** keratinocyte (NHK) growth in culture and as a function of sodium n-butyrate (NaB)-induced differentiation to mature enucleate cornified envelopes (CEs). Low levels of SPARC expression were observed in the basal-like cells of control NHKs, with isolated cells showing intense SPARC expression on the ventral surface. After addition of NaB, SPARC expression increased and the pattern of expression shifted to one involving predominantly suprabasal cells (i.e., spinous cells, pre-CEs, and mature CEs). Dense deposits of SPARC often surrounded the mature CEs. Flow cytometric analysis indicated that approximately 13% of NHKs expressed SPARC within 24 h of seeding into culture. This fraction of SPARC+ cells increased with time and peaked immediately postconfluence (31.3 +/- 6.3% SPARC+). Cellular SPARC expression then decreased to baseline levels during entrance into plateau phase growth. SPARC was detectable in all phases of the cell cycle. SPARC levels were more intense and heterogeneous within the G2/M and G1 phases while S phase cells exhibited relatively homogeneous, low intensity, SPARC expression. During NaB-induced NHK differentiation, SPARC intracellular content increased prior to the onset of CE formation (i.e., 2 days after its addition) followed by a period of extracellular accumulation which coincided with the time of maximal CE generation (i.e., Days 4 and 5 after NaB addition). Correlation of cell size with anti-SPARC immunoreactivity revealed a predominance of SPARC expression in cells with a suprabasal phenotype. NHKs cultured on fibronectin (FN), an established modulator of epidermal cell maturation in vitro, showed a similar response to NaB. In general, however, the level of NaB-induced SPARC expression was considerably reduced in FN cultures correlating with a lower efficiency of CE formation. Induced SPARC expression was, in large part, dependent on autocrine transforming growth factor-beta (TGF-beta) production since incubation in the presence of NaB+neutralizing antibodies to TGF-beta **inhibited** both the expression of SPARC by 72% and development of mature CEs.

6/3,AB/14 (Item 14 from file: 155)
DIALOG(R)File 155:MEDLINE(R)

07911358 93139938 PMID: 8380854
Calcification **inhibitors** in **human** ligamentum flavum.
Maruta K; Ichimura K; Matsui H; Yamagami T; Sano A; Tsuji H
Department of Orthopaedic Surgery, Faculty of Medicine, Toyama Medical and Pharmaceutical University, Japan.
Journal of orthopaedic research (UNITED STATES) Jan **1993**, 11 (1) p92-103, ISSN 0736-0266 Journal Code: JIQ
Languages: ENGLISH
Document type: Journal Article
Record type: Completed
To examine the presence of substances which **inhibit** calcification in **human** ligamentum flavum, the **inhibitory** effect of an Na2HPO4 extract of the flavum was determined in terms of the in vitro calcium uptake of the ligamentum flavum matrix. Additionally, grafts of extracted and non-extracted dry ligamentum flavum matrices were transplanted into the dorsal muscles of rats, and calcification in the grafts was examined radiologically and histochemically. In order to determine if component cells of **human** ligamentum flavum produce calcification **inhibitors**, ligamentum flavum cells were cultured, and the crystal **inhibitor** activity of the culture medium was measured by a seed test which used hydroxyapatite as the nucleus of precipitation. The calcification reaction system demonstrated that the ligamentum flavum extract contains an **inhibitory** factor for calcium uptake by the ligamentum flavum matrix. The seed test revealed that **human** ligamentum flavum cells produce calcification **inhibitor** activity.

6/3,AB/15 (Item 15 from file: 155)
DIALOG(R)File 155:MEDLINE(R)

07842316 91350026 PMID: 1831853
Induction of formative and resorptive cellular phenotypes in **human** gingival fibroblasts by TGF-beta 1 and concanavalin A: regulation of matrix metalloproteinases and TIMP.
Overall CM; Wrana JL; Sodek J
Department of Clinical Dental Sciences, Faculty of Dentistry, University of British Columbia, Vancouver, Canada.
Journal of periodontal research (DENMARK) May **1991**, 26 (3 Pt 2) p279-82, ISSN 0022-3484 Journal Code: JMQ
Languages: ENGLISH
Document type: Journal Article
Record type: Completed

6/3,AB/16 (Item 16 from file: 155)
DIALOG(R)File 155:MEDLINE(R)

07813838 93093130 PMID: 1281115
Isolation and characterization of gene sequences overexpressed in Werner syndrome fibroblasts during premature replicative senescence.
Thweatt R; Murano S; Fleischmann RD; Goldstein S
Department of Medicine, University of Arkansas for Medical Sciences, Little Rock.
Experimental gerontology (ENGLAND) Jul-Aug **1992**, 27 (4) p433-40, ISSN 0531-5565 Journal Code: EPQ
Contract/Grant No.: AG-05554, AG, NIA; AG-08708, AG, NIA
Languages: ENGLISH
Document type: Journal Article
Record type: Completed

6/3,AB/17 (Item 17 from file: 155)
DIALOG(R)File 155:MEDLINE(R)

07794286 92180696 PMID: 1796754
Osteonectin is an alpha-granule component involved with thrombospondin in platelet aggregation.
Clezardin P; Malaval L; Morel MC; Guichard J; Lecompte T; Trzeciak MC; Dechavanne M; Breton-Gorius J; Delmas PD; Kaplan C
INSERM U 234, Laboratoire de Biochimie des Proteines Osseuses, Hopital Edouard Herriot, Lyon, France.
Journal of bone and mineral research (UNITED STATES) Oct **1991**, 6 (10) p1059-70, ISSN 0884-0431 Journal Code: 130
Languages: ENGLISH
Document type: Journal Article
Record type: Completed
We previously showed that thrombospondin, a major alpha-granule glycoprotein of **human** platelets, forms a specific complex with **osteonectin** , a phosphoglycoprotein originally described in bone that is also present in **human** platelets. The storage organelles and the function of **osteonectin** in platelets are still unknown. In this study, using electron microscopy in combination with immunogold staining, the major storage organelle for platelet-secreted proteins, the alpha-granules. Furthermore, **osteonectin** was qualitatively and quantitatively assessed by studying normal platelets and the platelets from a patient with gray platelet syndrome. Gray platelet syndrome is a rare congenital bleeding disorder characterized by a selective deficiency in morphologically recognizable platelet alpha-granules and in the alpha-granule secretory proteins. Binding of an iodinated antiosteonectin monoclonal antibody to gray platelet proteins transferred to nitrocellulose

from SDS-polyacrylamide gels showed no band corresponding to **osteonectin** compared to control platelets. Using a polyclonal antiosteonectin antibody-based radioimmunoassay, gray platelets contained 0.2 +/- 0.03 ng **osteonectin** per 10(6) platelets, which is only 20% of the normal platelet content of **osteonectin** (0.93 +/- 0.16 ng per 10(6) platelets). Study of the localization of **osteonectin** to the surface of **human** platelets demonstrated that a radioiodinated antiosteonectin polyclonal antibody bound specifically to thrombin-stimulated platelets but not to resting platelets. Binding was concentration-dependent, saturable (1710 +/- 453 binding sites per platelet, Kd = 1 microM), and **inhibited** by an excess of cold antiosteonectin polyclonal antibody. No binding was observed on the surface of thrombin-stimulated gray platelets. To gain further insights into the role of **osteonectin** released from activated platelets, the effect of an antiosteonectin polyclonal antibody was tested on the aggregation of washed platelets. F(ab')2 fragments from the antiosteonectin polyclonal antibody **inhibited** in a dose-dependent manner the aggregation of collagen-stimulated, washed **human** platelets without affecting collagen-induced platelet serotonin release. To characterize the mechanism through which antiosteonectin F(ab')2 fragments **inhibit** platelet aggregation, the expression of endogenous thrombospondin (TSP) on the surface of thrombin-activated platelets was studied using 125I-labeled anti-TSP monoclonal antibody P10. The endogenous surface expression of TSP to thrombin-stimulated platelets was significantly **inhibited** in the presence of antiosteonectin F(ab')2 fragments (6286 +/- 2065 molecules of P10 per platelet) compared to 11,230 +/- 766 molecules of P10 per platelet in the presence of nonimmune F(ab')2 fragments.(ABSTRACT TRUNCATED AT 400 WORDS)

6/3,AB/18 (Item 18 from file: 155)
DIALOG(R)File 155:MEDLINE(R)

07776783 91304381 PMID: 1712899

Diverse gene sequences are overexpressed in werner syndrome fibroblasts undergoing premature replicative senescence.

Murano S; Thweatt R; Shmookler Reis RJ; Jones RA; Moerman EJ; Goldstein S
Departments of Medicine, University of Arkansas for Medical Sciences, Little Rock.

Molecular and cellular biology (UNITED STATES) Aug **1991**, 11 (8) p3905-14, ISSN 0270-7306 Journal Code: NGY
Contract/Grant No.: AG-08708, AG, NIA
Languages: ENGLISH
Document type: Journal Article
Record type: Completed

Genes that play a role in the senescent arrest of cellular replication are likely to be overexpressed in **human** diploid fibroblasts (HDF) derived from subjects with Werner syndrome (WS) because these cells have a severely curtailed replicative life span. To identify some of these genes, a cDNA library was constructed from WS HDF after they had been serum depleted and repleted (5 days in medium containing 1% serum followed by 24 h in medium containing 20% serum). Differential screening of 7,500 colonies revealed 102 clones that hybridized preferentially with [32P]cDNA derived from RNA of WS cells compared with [32P]cDNA derived from normal HDF. Cross-hybridization and partial DNA sequence determination identified 18 independent gene sequences, 9 of them known and 9 unknown. The known genes included alpha 1(I) procollagen, alpha 2(I) procollagen, fibronectin, ferritin heavy chain, insulinlike growth factor-binding protein-3 (IGFBP-3), **osteonectin**, **human** tissue plasminogen activator **inhibitor** type I, thrombospondin, and alpha B-crystallin. The nine unknown clones included two novel gene sequences and seven additional sequences that contained both novel segments and the Alu class of repetitive short interspersed nuclear elements; five of these seven Alu+ clones also contained the long interpersed nuclear element I (KpnI) family

of repetitive elements. Northern (RNA) analysis, using the 18 sequences as probes, showed higher levels of these mRNAs in WS HDF than in normal HDF. Five selected mRNAs studied in greater detail [alpha 1(I) procollagen, fibronectin, insulinlike growth factor-binding protein-3, WS3-10, and WS9-14] showed higher mRNA levels in both WS and late-passage normal HDF than in early-passage normal HDF at various intervals following serum depletion/repletion and after subculture and growth from sparse to high-density confluent arrest. These results indicate that senescence of both WS and normal HDF is accompanied by overexpression of similar sets of diverse genes which may play a role in the senescent arrest of cellular replication and in the genesis of WS, normal biological aging, and attendant diseases.

6/3,AB/19 (Item 19 from file: 155)
DIALOG(R)File 155:MEDLINE(R)

07682591 93130187 PMID: 1483113
Synthesis and secretion of glycosaminoglycans and proteins in **human** normal and otosclerotic bone cells.
Locci P; Venti G; Lilli C; Becchetti E; Paludetti G; Donti E; Marinucci L ; Maurizi M
Dipartimento di Medicina Sperimentale, Universita di Perugia, Italy.
Cellular and molecular biology (FRANCE) Aug-Sep **1992**, 38 (5-6) p635-42, ISSN 0145-5680 Journal Code: BNA
Languages: ENGLISH
Document type: Journal Article
Record type: Completed
Some biosynthetic activities of normal and otosclerotic temporal bone cultures have been studied. Bone cells were cultured for 24 hrs. in medium containing 3H-glucosamine, 35SO4 or 3H-proline. Labelled glycosaminoglycans (GAG) and proteins were precipitated from cells and media. In otosclerotic bone cells there was an evident reduction in the synthesis and secretion of radiolabelled macromolecules. The **inhibitory** effect was always greater in the extracellular than in the intracellular compartment. Some glycosidases were also studied. Otosclerosis decreased the activity of all enzymes examined, indicating that the lower GAG synthesis and secretion in otosclerotic bone cells were not due to an increased degradation.

6/3,AB/20 (Item 20 from file: 155)
DIALOG(R)File 155:MEDLINE(R)

07681652 93128501 PMID: 1481911
Antibodies to SPARC **inhibit** albumin binding to SPARC, gp60, and microvascular endothelium.
Schnitzer JE; Oh P
Department of Medicine and Pathology, School of Medicine, University of California, San Diego, La Jolla 92093-0651.
American journal of physiology (UNITED STATES) Dec **1992**, 263 (6 Pt 2) pH1872-9, ISSN 0002-9513 Journal Code: 3U8
Contract/Grant No.: HL-17080, HL, NHLBI; HL-43278, HL, NHLBI
Languages: ENGLISH
Document type: Journal Article
Record type: Completed
Albumin, through its binding to the endothelial glycocalyx, functions as a major determinant of capillary permeability and as a carrier for various small molecules in its transcytosis across continuous endothelium via plasma-lemmal vesicles. Several albumin-binding proteins (ABP) have been identified: three membrane-associated ABP, which we call gp60, gp30, and gp18, and one secreted protein, acidic and rich in cysteine (SPARC). In this study, we used antiserum raised against bovine SPARC (BON) to investigate the possible interrelationships among ABP to better understand their role in binding and transcytosis. BON not only interacted with SPARC

secreted by cultured endothelium but also recognized gp60 in lysates of cultured rat, **human**, and bovine endothelial cells. Purified SPARC **inhibited** BON interaction with gp60. BON immunoglobulin (Ig)G specifically **inhibited** albumin binding to both SPARC and gp60 extracts. This effect was eliminated by preabsorption of BON to immobilized SPARC. BON also significantly **inhibited** albumin binding to cultured microvascular endothelial cells via its interaction with gp60. Anti-SPARC peptide sera were also tested, and one serum raised against a peptide encompassing an NH2-terminal region of SPARC recognized both SPARC and gp60 but did not **inhibit** albumin binding; gp30 and gp18 were not recognized by any of these anti-SPARC antibodies. These results suggest that SPARC and gp60 are functionally and immunologically related ABP that may share a common albumin-binding domain. gp60 appears to be the major mediator of albumin binding to microvascular endothelium.

6/3,AB/21 (Item 21 from file: 155)
DIALOG(R)File 155:MEDLINE(R)

07606150 92312346 PMID: 1615759
Identification of proteins secreted by **human** osteoblastic cells in culture.
Johansen JS; Williamson MK; Rice JS; Price PA
Department of Biology, University of California-San Diego, La Jolla.
Journal of bone and mineral research (UNITED STATES) May **1992**, 7 (5) p501-12, ISSN 0884-0431 Journal Code: 130
Contract/Grant No.: AR 27029, AR, NIAMS
Languages: ENGLISH
Document type: Journal Article
Record type: Completed

To better understand the biochemistry of matrix-forming cells, we developed a simple and reproducible procedure for the isolation and identification by N-terminal sequencing of proteins secreted by cells into culture medium and applied this procedure to the analysis of the major Coomassie blue-staining proteins under 100 kD that are secreted from three different **human** osteoblastic cell cultures. The major proteins secreted by normal **human** osteoblasts from adult trabecular bone were identified by N-terminal sequencing to be gelatinase, **osteonectin**, the C-terminal propeptides of the alpha 1 and alpha 2 chains of type I collagen, tissue **inhibitor** of metalloproteinase 1 (TIMP-1), and beta 2-microglobulin. The amounts of each of these proteins secreted into medium over a 24 h interval did not change over the 7 consecutive days of culture under serum-free conditions, which indicates that this pattern of protein secretion is not significantly affected by the serum-free conditions needed for protein identification by this method. In addition, radioimmunoassay for bone gla protein (BGP), a marker for osteoblast phenotype, revealed that BGP secretion remained high over 7 days of culture under serum-free conditions and was comparable to the rate of BGP secretion in control cultures with 10% serum. The major proteins secreted by MG-63 cells were identified by N-terminal sequencing to be gelatinase, a novel 40 kD **human** bone protein we termed YKL-40, TIMP-1, the recently discovered TIMP-2, and beta 2-microglobulin. Further studies revealed that YKL-40 is the only protein detectable by Coomassie staining of SDS gels of MG-63 media proteins that is induced by extended time at confluence or by treatment with 1,25-(OH)2D3. The apparent absence of detectable Coomassie-stained bands corresponding to the C-terminal propeptides of collagen in the medium of MG-63 cells suggests that these transformed cells may not be a good model for bone matrix formation. The major proteins secreted by normal fetal osteoblastic cells were identified by N-terminal sequencing to be **osteonectin** and the C-terminal propeptides of the alpha 1 and alpha 2 chains of type I collagen. Gelatinase and TIMP could not be detected among the conditioned medium proteins by these methods. These observations indicate that fetal osteoblasts primarily express proteins that are matrix constituents and adult **human** osteoblasts

secrete, in addition to these, proteins that could function in matrix turnover.

6/3,AB/22 (Item 22 from file: 155)
DIALOG(R)File 155:MEDLINE(R)

07175019 92159033 PMID: 1311092

The extracellular glycoprotein SPARC interacts with platelet-derived growth factor (PDGF)-AB and -BB and **inhibits** the binding of PDGF to its receptors.

Raines EW; Lane TF; Iruela-Arispe ML; Ross R; Sage EH
Department of Pathology, University of Washington, Seattle 98195.

Proceedings of the National Academy of Sciences of the United States of America (UNITED STATES) Feb 15 **1992**, 89 (4) p1281-5, ISSN 0027-8424 Journal Code: PV3
Contract/Grant No.: GM40711, GM, NIGMS; HL18645, HL, NHLBI; RR-00166, RR, NCRR; +
Languages: ENGLISH
Document type: Journal Article
Record type: Completed

Interactions among growth factors, cells, and extracellular matrix are critical to the regulation of directed cell migration and proliferation associated with development, wound healing, and pathologic processes. Here we report the association of PDGF-AB and -BB, but not PDGF-AA, with the extracellular glycoprotein SPARC. Complexes of SPARC and 125I-labeled PDGF-BB or -AB were specifically immunoprecipitated by anti-SPARC immunoglobulins. 125I-PDGF-BB and -AB also bound specifically to SPARC that was immobilized on microtiter wells or bound to nitrocellulose after transfer from SDS/polyacrylamide gels. The binding of PDGF-BB to SPARC was pH-dependent; significant binding was detectable only above pH 6.6. The interaction of SPARC with specific dimeric forms of PDGF affected the activity of this mitogen. SPARC **inhibited** the binding of PDGF-BB and PDGF-AB, but not PDGF-AA, to **human** dermal fibroblasts in a dose-dependent manner. The expression of SPARC and PDGF was minimal in most normal adult tissues but was increased after injury. Enhanced expression of both PDGF-B chain and SPARC was seen in advanced lesions of atherosclerosis. We suggest that the coordinate expression of SPARC and PDGF-B-containing dimers following vascular injury may regulate the activity of specific dimeric forms of PDGF in vivo.

6/3,AB/23 (Item 23 from file: 155)
DIALOG(R)File 155:MEDLINE(R)

07079843 93387262 PMID: 8397080

Extracellular protein secretion of cultured normal and Pagetic osteoblasts.

Hankey DP; Hughes AE; Mollan RA; Nicholas RM
School of Biomedical Sciences/Anatomy, Queen's University of Belfast, Northern Ireland.

Electrophoresis (GERMANY) Jul **1993**, 14 (7) p644-9, ISSN 0173-0835 Journal Code: ELE
Languages: ENGLISH
Document type: Journal Article
Record type: Completed

Two-dimensional polyacrylamide gel electrophoresis (2-D PAGE) has been used to map the extracellular secretory activity of normal osteoblasts. The proteins **osteonectin** , bone sialoprotein, and both the C-telopeptide of collagen I together with collagen I have now been positionally identified. In addition the secretory differences which exist between normal and Pagetic osteoblasts have been mapped with the Pagetic osteoblasts shown to consistently secrete an altered 30 kDa C-telopeptide of collagen type I. The use of the diphosphonate Pamidronate in the

treatment of Paget's disease of bone has beneficial effects with suppression of the bone isoenzyme marker alkaline phosphatase. It has been reported that diphosphonates directly **inhibit human** osteoblast secretory function as well as osteoclast metabolism. The effects of Pamidronate on the secretory activity of normal and Pagetic osteoblast cultures was also investigated. The extracellular protein secretion of normal and Pagetic osteoblasts was not affected by Pamidronate treatment as assessed by 2-D PAGE. This technique allows a comprehensive multiparameter assessment of extracellular secretory activity in normal and diseased states. The findings show that the Pagetic osteoblasts cultured in vitro are functionally abnormal and they support the hypothesis that the underlying problem in Paget's disease is characterised by disorder of osteoblast and osteoclast interactions.

6/3,AB/24 (Item 24 from file: 155)
DIALOG(R)File 155:MEDLINE(R)

07026052 93132085 PMID: 8421066
Structure and binding properties of collagen type XIV isolated from **human** placenta.
Brown JC; Mann K; Wiedemann H; Timpl R
Max-Planck-Institut fur Biochemie, Martinsried, Germany.
Journal of cell biology (UNITED STATES) Jan **1993**, 120 (2) p557-67, ISSN 0021-9525 Journal Code: HMV
Languages: ENGLISH
Document type: Journal Article
Record type: Completed
Collagen XIV was isolated from neutral salt extracts of **human** placenta and purified by several chromatographic steps including affinity binding to heparin. The same procedures also led to the purification of a tissue form of fibronectin. Collagen XIV was demonstrated by partial sequence analysis of its Col1 and Col2 domains and by electron microscopy to be a disulphide-linked molecule with a characteristic cross-shape. The individual chains had a size of approximately 210 kD, which was reduced to approximately 180 kD (domain NC3) after treatment with bacterial collagenase. Specific antibodies mainly to NC3 epitopes were obtained by affinity chromatography and used in tissue and cell analyses by immunoblotting and radioimmunoassays. Two sequences from NC3 were identified on fragments obtained after trypsin cleavage. They were identical to cDNA-derived sequences of undulin, a noncollagenous extracellular matrix protein. This suggests that collagen XIV and undulin may be different splice variants from the same gene. Heparin binding was confirmed in ligand assays with a large basement membrane heparan sulphate proteoglycan. This binding could be **inhibited** by heparin and heparan sulphate but not by chondroitin sulphate. In addition, collagen XIV bound to the triple helical domain of collagen VI. The interactions with heparin sulphate proteoglycan and collagen VI were not shared by the NC3 domain, or by reduced and alkylated collagen XIV. No or only low binding was observed for collagens I-V, pN-collagens I and III, and several noncollagenous matrix proteins, including laminin, recombinant nidogen, BM-40/ **osteonectin** , plasma and tissue fibronectin, vitronectin, and von Willebrand factor. Insignificant activity was also shown in cell attachment assays with nine established cell lines.

6/3,AB/25 (Item 25 from file: 155)
DIALOG(R)File 155:MEDLINE(R)

06844127 91224147 PMID: 1709099
Regulation of the expression of a secreted acidic protein rich in cysteine (SPARC) in **human** fibroblasts by transforming growth factor beta. Comparison of transcriptional and post-transcriptional control with fibronectin and type I collagen.

Wrana JL; Overall CM; Sodek J
Department of Biochemistry, University of Toronto, Canada.
European journal of biochemistry (GERMANY) Apr 23 **1991**, 197 (2) p519-28, ISSN 0014-2956 Journal Code: EMZ
Languages: ENGLISH
Document type: Journal Article
Record type: Completed
Transforming growth factor beta (TGF-beta) and secreted protein acidic rich cysteine (SPARC) have been associated with the rapid remodeling of connective tissues that occurs in wound healing and developmental processes. To study the temporal and mechanistic aspects of TGF-beta-regulated extracellular-protein gene expression in **human** fibroblasts, confluent cells were pulse labeled for 30 min with [35S]methionine at various times following the single addition of 1.0 ng/ml TGF-beta. After a 4-h chase period, specific radiolabeled media proteins were isolated by either immunoprecipitation or affinity chromatography and quantitated. Stimulation of SPARC synthesis was first apparent 5 h after addition of TGF-beta, reached a maximum (3.5-fold increase) at 24 h and persisted for at least 96 h. A similar temporal response to TGF-beta was observed for the extracellular matrix proteins collagen and fibronectin. In contrast, TGF-beta induced a strong (greater than sixfold increase at 9 h after addition of TGF-beta), but transient stimulation of the synthesis of endothelial-type plasminogen activator **inhibitor** . Northern blot analysis showed that SPARC mRNA levels were increased by TGF-beta in parallel with increase in SPARC synthesis; a maximum 3.9-fold increase in SPARC mRNA being reached at 24 h. Similarly, the levels of both collagen and fibronectin mRNA were increased by TGF-beta treatment. In each case the stimulation of mRNA was blocked by the presence of the translation **inhibitor** , cycloheximide. Stability of SPARC mRNA (half-life of approximately 50 h) was not significantly altered by TGF-beta. In contrast, the stability of collagen and fibronectin mRNA were both increased in the presence of TGF-beta; the increased stability being pronounced in less dense cells. In addition to effects on stability, transcription of the collagen and fibronectin genes was increased 7 h after TGF-beta addition, but returned to control levels by 24 h. However, transcription of the SPARC gene was unaffected by TGF-beta at both time points and, together with the stability data, indicates that TGF-beta regulates SPARC expression via a nuclear post-transcriptional mechanism. Differential regulation of gene expression by TGF-beta in a precise temporal pattern via transcriptional and post-transcriptional pathways may be an important aspect of the response of fibroblast cells in a wound environment.

6/3,AB/26 (Item 26 from file: 155)
DIALOG(R)File 155:MEDLINE(R)

06751053 91249758 PMID: 2040255

Physiology of bone: mineral compartment proteins as candidates for environmental perturbation by lead.

Sauk JJ; Somerman MJ
Department of Pathology, University of Maryland Dental School, Baltimore 21201.
Environmental health perspectives (UNITED STATES) Feb **1991**, 91 p9-16, ISSN 0091-6765 Journal Code: EI0
Contract/Grant No.: DE07512, DE, NIDCR
Languages: ENGLISH
Document type: Journal Article; Review; Review, Tutorial
Record type: Completed
Termine et al. first demonstrated that sequential dissociative extraction and fractionation procedures with protease **inhibitors** could provide a convenient approach for the study of mineral compartment constituents. The primary extraction regimen used 4 M guanidine HCl to remove most of the protein from the nonmineralized phase of bone. Subsequently, EDTA-guanidine was used to remove the mineral-phase components. These methods discriminate

on the basis of physical-chemical association with a mineral phase rather than on the specific gene products of a particular cell. In the present discussion emphasis is directed at a group of divalent cation binding proteins isolated from the mineral compartment of bone. The localization, synthesis, and chemical characteristics of **osteonectin** , bone sialoproteins I and II, and bone acidic glycoprotein-75 are discussed and offered as possible sites for perturbation by the environment with lead exposure.

6/3,AB/27 (Item 27 from file: 155)
DIALOG(R)File 155:MEDLINE(R)

05931851 88296491 PMID: 3402455
Complex formation of **human** thrombospondin with **osteonectin**.
Clezardin P; Malaval L; Ehrensperger AS; Delmas PD; Dechavanne M; McGregor JL
Institut National de la Sante et de la Recherche Medicale, Faculte de Medecine Alexis-Carrel, Lyon, France.
European journal of biochemistry (GERMANY, WEST) Aug 1 **1988**, 175 (2) p275-84, ISSN 0014-2956 Journal Code: EMZ
Languages: ENGLISH
Document type: Journal Article
Record type: Completed
Human thrombospondin, a 450-kDa glycoprotein isolated from platelets and endothelial cells, specifically interacts with **osteonectin**, a protein of 30 kDa isolated from bovine bones and **human** platelets. Using ELISA, purified **osteonectin** binds to solid-phase-adsorbed thrombospondin with a dissociation constant (Kd) of 0.7 nM. Binding of thrombospondin to solid-phase-adsorbed **osteonectin** was also observed (Kd = 0.86 nM). The interaction of thrombospondin with solid-phase-adsorbed **osteonectin** was significantly decreased (81% **inhibition**) when using an excess of fluid-phase **osteonectin**. Thrombospondin-**osteonectin** complex formation was calcium-dependent as shown by a 50-80% **inhibition** in the presence of EDTA. None of the proteins known to interact with thrombospondin (fibrinogen, fibronectin, collagen, plasminogen) had a significant **inhibitory** effect on thrombospondin-**osteonectin** complex formation. This selective interaction was confirmed by affinity chromatography. Iodinated **osteonectin** , previously incubated with purified thrombospondin, specifically bound to an anti-thrombospondin monoclonal antibody (P10) linked to protein-A--Sepharose 4B. Elution of the anti-thrombospondin antibody from protein A allowed the recovery of the thrombospondin-**osteonectin** complex in the eluate, as judged by SDS/polyacrylamide gel electrophoresis and autoradiography. Blotting of purified thrombospondin to **osteonectin** adsorbed onto nitrocellulose further confirmed complex formation. In addition, when released from thrombin-stimulated platelets, thrombospondin and **osteonectin** bound to anti-thrombospondin IgG-coated plates indicating that **osteonectin** was complexed to thrombospondin once the platelet-release reaction has occurred.

6/3,AB/28 (Item 28 from file: 155)
DIALOG(R)File 155:MEDLINE(R)

05743874 86187762 PMID: 3008822
Inhibition of hydroxyapatite crystal growth by bone-specific and other calcium-binding proteins.
Romberg RW; Werness PG; Riggs BL; Mann KG
Biochemistry (UNITED STATES) Mar 11 **1986**, 25 (5) p1176-80, ISSN 0006-2960 Journal Code: A0G
Contract/Grant No.: AG04875, AG, NIA; AM07147, AM, NIADDK; AM20605, AM, NIADDK

Languages: ENGLISH
Document type: Journal Article
Record type: Completed
Mineralization of bone matrix may be influenced by the presence of specific, noncollagenous bone proteins. The quantitative influence of two bone-specific proteins--bone gamma-carboxyglutamic acid (Gla) protein and **osteonectin** --and other proteins that decreased the rate of crystal growth was measured by adding seed crystals of hydroxyapatite to a solution of CaCl2 and KH2PO4, pH 7.4 at 37 degrees C. The molar concentrations of proteins needed to **inhibit** the rate of crystal growth by 50% were as follows: **osteonectin** , 0.15 microM; bone Gla protein, 0.8 microM; prothrombin, 0.9 microM; prothrombin fragment 1, 1.0 microM; soybean trypsin **inhibitor** , 3 microM; prethrombin 1, 9 microM; cytochrome c, 30 microM. Calmodulin and parvalbumin were found to be less active than prothrombin fragment 1 and had no activity in the micromolar range. The combination of two **inhibitors** resulted in a mixture with an **inhibitory** activity that was the sum of the two **inhibitors**. Decarboxylation of bone Gla protein significantly reduced its **inhibitory** activity. These results indicate that the **inhibitory** activity of a protein does not correlate with Ca2+-binding affinity under these conditions, that the mixture of **inhibitors** has an additive effect, and that gamma-carboxyglutamic acid residues enhance the ability of a protein to **inhibit** hydroxyapatite-seeded crystal growth.

6/3,AB/29 (Item 29 from file: 155)
DIALOG(R)File 155:MEDLINE(R)

05616737 89120805 PMID: 3065073
Serum and urinary markers of bone remodeling: assessment of bone turnover.
Epstein S
Division of Endocrinology and Metabolism, Albert Einstein Medical Center, Philadelphia, Pennsylvania 19141.
Endocrine reviews (UNITED STATES) Nov **1988**, 9 (4) p437-49,
ISSN 0163-769X Journal Code: EIK
Languages: ENGLISH
Document type: Journal Article; Review; Review, Tutorial
Record type: Completed
It appears that at present, serum BGP is the one bone protein that has the most promise for assisting in the diagnosis and management of high turnover metabolic bone disease states. If further studies confirm its usefulness in osteoporosis as a predictor of rapid bone loss without the need for bone biopsy, this serum marker will then not only allow early detection but also an appropriate choice of therapy in osteoporosis, i.e. the use of specific **inhibitors** of high turnover states such as estrogen, calcitonin, or bisphosphonates. In addition, it may also permit more accurate follow-up of patients suffering from diseases such as primary hyperparathyroidism after surgery. In low turnover osteoporosis, it may also serve a useful function to observe whether the osteoblast can be stimulated to enhance bone formation with therapies such as fluoride, anabolic steroids, PTH, etc. As yet, additional measurements, such as bone histomorphometry and other bone mineral markers, are required for definitive diagnosis. Hopefully, the availability of specific well-characterized antibodies against BGP may define its role more accurately. Recently, several other new bone proteins have been identified but at present they have very limited clinical application. Future studies into the structure-function relationship of these bone proteins may identify those markers which will be most relevant to the diagnosis and treatment of metabolic bone disease.

6/3,AB/30 (Item 30 from file: 155)
DIALOG(R)File 155:MEDLINE(R)

05607172 89152544 PMID: 3068009
Non-collagen proteins in bone.
Termine JD
National Institute of Dental Research, Bethesda, Maryland 20892.
Ciba Foundation symposium (NETHERLANDS) **1988**, 136 p178-202,
ISSN 0300-5208 Journal Code: D7X
Languages: ENGLISH
Document type: Journal Article; Review; Review, Tutorial
Record type: Completed
The non-collagen proteins of bone are a complex set of molecules that arise from local or exogenous sources. Because bone mineral is an excellent adsorbent, many circulatory and/or cell surface proteins bind to bone, where they may have immediate or subsequent effects. These include the alpha 2-HS-glycoprotein from blood and the potent growth factors TGF-beta, PDGF, IGF-1, FGF-a and -b, and IL-1, derived from both bone and non-bone cells. Furthermore, bone cell membrane proteins such as alkaline phosphatase may be cleaved from the cell surface and entrapped in the bone matrix. Bone is enriched in a variety of enzymes and their **inhibitors** by similar adsorption processes. Even osteocalcin, a bone cell product, is adsorbed to bone via mineral-binding (Gla) groups. The bone sialoproteins (BSP-I or osteopontin and BSP-II) also bind to the mineral via acidic groups. Because of this phenomenon it is difficult to distinguish whether a given protein's presence in bone is advantageous or merely fortuitous. The bone matrix proper consists of type I collagen and other osteoblast products such as **osteonectin** (a phosphorylated glycoprotein) and small proteoglycans (PG-I and/or PG-II) which are incorporated into bone collagen fibrils. These proteins may have additional roles in tissue morphogenesis and/or differentiation.

6/3,AB/31 (Item 31 from file: 155)
DIALOG(R)File 155:MEDLINE(R)

05462098 89151095 PMID: 2493327
Osteonectin inhibiting de novo formation of apatite in the presence of collagen.
Doi Y; Okuda R; Takezawa Y; Shibata S; Moriwaki Y; Wakamatsu N; Shimizu N ; Moriyama K; Shimokawa H
Department of Dental Materials and Technology, School of Dentistry, Asahi University, Gifu, Japan.
Calcified tissue international (GERMANY, WEST) Mar **1989**, 44 (3) p200-8, ISSN 0171-967X Journal Code: CGH
Languages: ENGLISH
Document type: Journal Article
Record type: Completed
The effect of bone matrix protein of **osteonectin** on de novo formation of apatite was studied in a wide range of calcium phosphate solutions in the presence of collagen. In every solution, from which amorphous calcium phosphate, octacalcium phosphate, or apatite precipitated as a possible initial phase, **osteonectin** at concentrations less than 1 microM retarded the precipitation, subsequent transformation to apatite, and ripening crystal growth of apatite. Collagen present as either reconstituted or denatured form had no effect on the **osteonectin** -associated reactions as well as **osteonectin**-free reactions, and no structural correlation was observed between collagen fibrils and any of the calcium phosphates that appeared in our system. Direct measurement of free calcium levels in the solutions suggested that the reduction in calcium activity due to complexing with **osteonectin** hardly explained the **inhibitory** activity of **osteonectin** in retarding the formation of apatite. Instead, our transmission electron microscopic (TEM) observation strongly suggested that the primary mechanism for **osteonectin** to **inhibit** the formation of apatite is to block growth sites of calcium phosphates nucleated. The apatite thus formed in

the presence of **osteonectin** showed less resolved X-ray diffraction patterns, partly because of smaller crystallites as suggested by TEM.

6/3,AB/32 (Item 32 from file: 155)
DIALOG(R)File 155:MEDLINE(R)

05385757 89179899 PMID: 2648065
Mechanism of mineral formation in bone.
Anderson HC
Department of Pathology and Oncology, University of Kansas Medical Center, Kansas City.
Laboratory investigation (UNITED STATES) Mar **1989**, 60 (3) p320-30, ISSN 0023-6837 Journal Code: KZ4
Contract/Grant No.: DE05262, DE, NIDCR
Languages: ENGLISH
Document type: Journal Article; Review; Review, Academic
Record type: Completed
The mechanism of mineral formation in bone is seen best where active new bone formation is occurring, e.g., in newly forming subperiosteal bone of the embryo, in the growing bone of young animals, and in healing rickets where the calcification process in osteoid is reactivated. A large body of ultrastructural evidence, using conventional and anhydrous methods for tissue preparation, has shown convincingly that extracellular matrix vesicles are present at or near the mineralization front in all of the above, and that these vesicles are the initial site of apatite mineral deposition. Thus bone resembles growth plate cartilage, predentin, and turkey tendon in having calcification initiated by matrix vesicles. Once the calcification cascade is begun, matrix vesicles are no longer needed to support mineralization and are consumed by the advancing mineralization front in which performed crystals serve as nuclei for the formation of new crystals. The rate of crystal proliferation is promoted by the availability of Ca2+, PO4(3-), and the presence of collagen, and retarded by naturally occurring **inhibitors** of mineralization such as proteoglycans and several noncollagenous calcium-binding proteins of bone including bone-Gla protein (osteocalcin), phosphoproteins, **osteonectin** , and alpha-2HS-glycoproteins. New electron microscopic immunocytochemical findings in our laboratory suggest that the origin of alkaline phosphatase-positive bone matrix vesicles is polarized to the mineral-facing side of osteoblasts and may be concentrated near the intercellular junctions of **human** embryonic osteoblasts.

6/3,AB/33 (Item 33 from file: 155)
DIALOG(R)File 155:MEDLINE(R)

05316044 90001202 PMID: 2790009
Structure of **human osteonectin** based upon analysis of cDNA and genomic sequences.
Villarreal XC; Mann KG; Long GL
Department of Biochemistry, University of Vermont, Burlington 05405.
Biochemistry (UNITED STATES) Jul 25 **1989**, 28 (15) p6483-91, ISSN 0006-2960 Journal Code: A0G
Contract/Grant No.: C06 HL39745, HL, NHLBI; P01 AG04875, AG, NIA; R01 HL38899, HL, NHLBI
Languages: ENGLISH
Document type: Journal Article
Record type: Completed
Overlapping **human** bone **osteonectin** cDNAs were obtained by screening two independent **human** SaOS-2 lambda gt11 libraries using antibovine **osteonectin** monoclonal antibodies. One clone contains a 0.54-kb insert and the other a 1.9-kb insert. Insertion fragments from lambda clones were liberated by restriction digestion and subcloned into pUC19 for sequencing. Digestion of the 1.9-kb insert with EcoRI released

membrane, following close contacts between epithelial and fibroblastic cells, is necessary for the expression of differentiation markers such as digestive enzymes.

2/3,AB/5 (Item 5 from file: 155)
DIALOG(R)File 155:MEDLINE(R)

07721153 93228971 PMID: 1299273
Overexpression of SPARC in stably transfected F9 cells mediates attachment and spreading in Ca(2+)-deficient medium.
Everitt EA; Sage EH
Department of Biological Structure, School of Medicine, University of Washington, Seattle 98195.
Biochemistry and cell biology (CANADA) Dec 1992, 70 (12) p1368-79, ISSN 0829-8211 Journal Code: ALR
Contract/Grant No.: 5T32-GM07270, GM, NIGMS; GM-40711, GM, NIGMS
Languages: ENGLISH
Document type: Journal Article
Record type: Completed
The Ca(2+)-binding protein SPARC is one of a group of proteins that function in vitro to promote the rounding of cells. To assess whether the modulation of cell shape by SPARC is affected by extracellular Ca2+, we used F9 cell lines that had been stably transfected with sense or **antisense** SPARC DNA. Sense-transfected (S) lines that overexpress SPARC are aggregated and rounded, whereas **antisense** (AS) lines that express low levels of the protein are flat and spread. We tested whether the cell lines would exhibit these altered morphologies in Ca(2+)-deficient media. When cultured under these conditions, S lines attached and spread, whereas AS lines attached but remained round, with no subsequent spreading. Addition of CaCl2 or purified SPARC to the Ca(2+)-deficient medium resulted in spreading of the AS and control lines and a reappearance of the altered morphologies. Expression of the Ca(2+)-binding cadherin uvomorulin by the cell lines correlated with neither their morphology nor their level of SPARC expression. We conclude that the altered phenotypes of the transected lines reflect, in part, the concentration of extracellular Ca2+ and that the spreading exhibited by the S lines under Ca(2+)-deficient conditions is directly related to their enhanced expression of SPARC. SPARC might, therefore, mediate interactions between cells and matrix that are permissive for adhesion when levels of extracellular Ca2+ are diminished.

2/3,AB/6 (Item 1 from file: 5)
DIALOG(R)File 5:Biosis Previews(R)

11958706 BIOSIS NO.: 199900204815
Modulatory proteins and processes in alliance with immune cells, mediators, and extracellular proteins in renal interstitial fibrosis.
AUTHOR: Wardle E Nigel(a)
AUTHOR ADDRESS: (a)21 Common Road, North Leigh, Oxford, OX8 6RD**UK
JOURNAL: Renal Failure 21 (2):p121-133 March, 1999
ISSN: 0886-022X
DOCUMENT TYPE: Literature Review
RECORD TYPE: Abstract
LANGUAGE: English
SUMMARY LANGUAGE: English

ABSTRACT: A synopsis of the many aspects and factors that contribute to renal tubulo-interstitial fibrosis is presented. The role of fibrogenic cytokines and the conversion of fibroblasts to myofibroblasts are described. It is emphasized that oxygen radicals cause fibroblasts to generate collagen. The properties of those accessory modulatory proteins that affect the behavior of cells in the interstitium are considered and

how matrix for ensuing fibrosis is laid down. Understanding the extracellular matrix proteins and these modulatory proteins is important because their behavior can now be modified by means of **antisense** oligonucleotides.

12759369 BIOSIS NO.: 200000512992
The role of **SPARC** gene in human melanoma progression and as a target for gene therapy.
AUTHOR: Podhajcer Osvaldo L(a)
AUTHOR ADDRESS: (a)Gene Therapy Laboratory, Fundacion Campornar-CONICET, University of Buenos Aires, Buenos Aires**Argentina
JOURNAL: Pigment Cell Research 13 (3):p197-198 June, 2000
MEDIUM: print
CONFERENCE/MEETING: Ninth Meeting of the Pan American Society for Pigment Cell Research College Station, Texas, USA June 25-28, 2000
ISSN: 0893-5785
RECORD TYPE: Citation
LANGUAGE: English
SUMMARY LANGUAGE: English
2000

2/3,AB/7 (Item 2 from file: 5)
DIALOG(R)File 5:Biosis Previews(R)

08862815 BIOSIS NO.: 199396014316
Overexpression of **SPARC** in stably transfected F9 cells mediates attachment and spreading in calcium-deficient medium.
AUTHOR: Everitt Elizabeth A; Sage E Helene(a)
AUTHOR ADDRESS: (a)Dep. Biological Structure, Sch. Med., University Washington, Seattle, WA 98195
JOURNAL: Biochemistry and Cell Biology 70 (12):p1368-1379 1992
ISSN: 0829-8211
DOCUMENT TYPE: Article
RECORD TYPE: Abstract
LANGUAGE: English
SUMMARY LANGUAGE: English; French

ABSTRACT: The Ca-2+-binding protein **SPARC** is one of a group of proteins that function in vitro to promote the rounding of cells. To assess whether the modulation of cell shape of **SPARC** is affected by extracellular Ca-2+, we used F9 cell lines that had been stably transfected with sense or **antisense** **SPARC** DNA. Sense-transfected (S) lines that overexpress **SPARC** are aggregated and rounded, whereas **antisense** (AS) lines that express low levels of the protein ae flat and spread. We tested whether the cell lines would exhibit these altered morphologies in Ca-2+-deficient media. When cultured under these conditions, S lines attached and spread, whereas AS lines attached but remained round, with no subsequent spreading. Addition of CaCl-2 or purified **SPARC** to the Ca-2+-deficient medium resulted in spreading of the AS and control lines and a reappearance of the altered morphologies. Expression of the Ca-2+-binding cadherin uvomorulin by the cell lines correlated with neither their morphology nor their level of **SPARC** expression. We conclude that the altered phenotypes of the transected lines reflect, in part, the concentration of extracellular Ca-2+ and that the spreading exhibited by the S lines under Ca-2+-deficient conditions is directly related to their enhanced expression of **SPARC**. **SPARC** might, therefore, mediate interactions between cells and matrix that are permissive for adhesion when levels of extracellular Ca-2+ are diminished.

1992

2/3,AB/8 (Item 3 from file: 5)
DIALOG(R)File 5:Biosis Previews(R)

Demonstration of endogenous **inhibitor** of PDGF (SPARC/ **Osteonectin**) gene expression in glomeruli in **human** proliferative glomerulonephritis by in situ.
AUTHOR: Yoshimura Ashio; Inui Kiyoko; Uda Susumu; Nabeshima Kunihiro; Taira Takayasu; Iwasaki Shigeki; Ideura Terukuni
AUTHOR ADDRESS: Showa Univ. Fujigaoka Hosp., Yokohama**Japan
JOURNAL: Journal of the American Society of Nephrology 5 (3):p847
1994
CONFERENCE/MEETING: Abstracts Submitted for the 27th Annual Meeting of the American Society of Nephrology Orlando, Florida, USA October 26-29, 1994
ISSN: 1046-6673
RECORD TYPE: Citation
LANGUAGE: English
1994

6/3,AB/36 (Item 3 from file: 5)
DIALOG(R)File 5:Biosis Previews(R)

08932402 BIOSIS NO.: 199396083903
TIMP-02, a growth-stimulatory protein from SV40-transformed **human** fibroblasts.
AUTHOR: Nemeth Jeffrey A; Goolsby Charles L(a)
AUTHOR ADDRESS: (a)Northwestern Univ. Med. Sch., Ward 6-204, 303 East Chicago Ave., Chicago, IL 60611**USA
JOURNAL: Experimental Cell Research 207 (2):p376-382 **1993**
ISSN: 0014-4827
DOCUMENT TYPE: Article
RECORD TYPE: Abstract
LANGUAGE: English

ABSTRACT: The mechanisms by which the early genes of simian virus 40 (SV40) transform **human** cells are unclear; however, this is clearly a multistep process involving a number of cellular and genetic changes. An early change following expression of the SV40 genes is growth under reduced serum conditions, which could be consistent with the production of autocrine/paracrine growth factors. HSF4-T12 is a **human** fibroblast cell line produced by transfection of primary cells with the genes for large T and small t antigens. A progressive stepwise transformation was observed with in vitro culture, eventually resulting in a tumorigenic cell line. Serum-free defined medium conditioned by HSF4-T12 was able to stimulate growth of normal **human** fibroblasts as determined by growth curve and (3H)thymidine incorporation assays. Purification of this activity by heparin affinity chromatography and nondenaturing polyacrylamide gel electrophoresis resulted in a single band of approximately 21 kDa on a nonreducing, denaturing gel. A partial 14-amino acid sequence was found to share 100% homology with a region of tissue **inhibitor** of metalloproteinases-2 (TIMP-2). Western blot analysis with anti-TIMP-2 antiserum confirmed this identification, and addition of this same antiserum to HSF4-T12-conditioned medium resulted in **inhibition** of stimulatory activity.

1993

6/3,AB/37 (Item 4 from file: 5)
DIALOG(R)File 5:Biosis Previews(R)

08885196 BIOSIS NO.: 199396036697
Skeletal unloading in rat decreases proliferation of rat bone and marrow-derived osteoblastic cells.
AUTHOR: Machwate Mohamed; Zerath Erik; Holy Xavier; Hott Monique; Modrowski

0.4- and 1.5-kb fragments. Sequencing analysis revealed that the 0.54- and 0.4-kb fragments are identical except for 150 nucleotides missing at the 5' region of the 0.4-kb fragment. The composite nucleotide sequence of **human osteonectin** has a total length of 2091 nucleotides and is comprised of 50 nucleotides of 5'-noncoding sequence, a coding segment for 303 amino acids, a termination codon, and 1114 nucleotides of 3'-noncoding sequence. The primary transcript codes for 286 amino acids of mature protein and a 17-residue amino-terminal hydrophobic signal peptide. Outstanding properties inferred from the primary structure are putative Ca2+ binding domains located in the glutamic acid rich NH2 terminus (residues 1-52) and two "EF"-hand domains in the C-terminal half of the protein (residues 165-176 and 257-286). The mature protein also contains a cysteine-rich, highly hydrophilic region homologous to the ovomucoid serine protease **inhibitors** (residues 76-132). Overlapping **human** genomic clones in lambda EMBL3 for **osteonectin** have been isolated and characterized. Intron/exon junction sequencing of the **human osteonectin** gene shows the presence of 10 exons and 9 introns. The mature protein is encoded by nine exons separated by eight introns.(ABSTRACT TRUNCATED AT 250 WORDS)

6/3,AB/34 (Item 1 from file: 5)
DIALOG(R)File 5:Biosis Previews(R)

09814322 BIOSIS NO.: 199598269240
Phenotypic modification of **human** osteosarcoma cells with the phorbol ester 12-O-tetradecanoylphorbol-13-acetate.
AUTHOR: Ringbom-Anderson Tove(a); Sandberg Minna; Andersson Goran; Akerman Karl E O
AUTHOR ADDRESS: (a)Dep. Biochem. Pharmacy, Abo Akad. Univ., PO Box 66, 20520 Turku**Finland
JOURNAL: Cell Growth & Differentiation 6 (4):p457-464 **1995**
ISSN: 1044-9523
DOCUMENT TYPE: Article
RECORD TYPE: Abstract
LANGUAGE: English

ABSTRACT: Treatment of the U-2 OS **human** osteosarcoma cell line with the phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA) dramatically decreased the rate of DNA synthesis. This decrease in proliferation as well as the change in morphology of the TPA-treated cells can be blocked by the protein kinase C **inhibitor** GF 109203X. The U-2 OS cells are known to express the c-sis oncogene (platelet-derived growth factor (PDGF) B-chain), PDGF-A, and receptors for PDGF, thus providing a potential autocrine loop of growth stimulation. TPA was found to induce the expression of both the PDGF-A and the PDGF-B chains. However, the levels of the PDGF receptor beta subunits and of the PDGF-BB inducible tyrosine phosphorylation of the PDGF receptor were markedly reduced. The TPA treatment of the U-2 OS cells also induced changes typical for maturing bone cells, such as increased expression levels of alkaline phosphatase and osteopontin. The expression levels of type I collagen and bone sialoprotein were reduced. The results show a TPA-dependent down-regulation of the PDGF receptor beta subunits that correlates with an increased expression of osteoblast phenotypic markers.

1995

6/3,AB/35 (Item 2 from file: 5)
DIALOG(R)File 5:Biosis Previews(R)

09523820 BIOSIS NO.: 199497532190

AS) lines that express low levels of the protein are flat and spread. We tested whether the cell lines would exhibit these altered morphologies in Ca 2+ deficient media. When cultured under these conditions, S lines attached and spread, whereas AS lines attached but remained round, with no subsequent spreading. Addition of CaCl2 or purified **SPARC** to the Ca 2+ -deficient medium resulted in spreading of the AS and control lines and a reappearance of the altered morphologies. Expression of the Ca 2+ -binding cadherin uvomorulin by the cell lines correlated with neither their morphology nor their level of **SPARC** expression. We conclude that the altered phenotypes of the transected lines reflect, in part, the concentration of extracellular Ca2+ and that the spreading exhibited by the S lines under Ca 2+ -deficient conditions is directly related to their enhanced expression of **SPARC**. **SPARC** might, therefore, mediate interactions between cells and matrix that are permissive for adhesion when levels of extracellular Ca2+ are diminished.

3/3,AB/6 (Item 1 from file: 5)
DIALOG(R)File 5:Biosis Previews(R)

08862815 BIOSIS NO.: 199396014316
Overexpression of **SPARC** in stably transfected F9 cells mediates attachment and spreading in calcium-deficient medium.
AUTHOR: Everitt Elizabeth A; Sage E Helene(a)
AUTHOR ADDRESS: (a)Dep. Biological Structure, Sch. Med., University Washington, Seattle, WA 98195
JOURNAL: Biochemistry and Cell Biology 70 (12):p1368-1379 **1992**
ISSN: 0829-8211
DOCUMENT TYPE: Article
RECORD TYPE: Abstract
LANGUAGE: English
SUMMARY LANGUAGE: English; French

ABSTRACT: The Ca-2+-binding protein **SPARC** is one of a group of proteins that function in vitro to promote the rounding of cells. To assess whether the modulation of cell shape of **SPARC** is affected by extracellular Ca-2+, we used F9 cell lines that had been stably transfected with sense or **antisense SPARC** DNA.
Sense-transfected (S) lines that overexpress **SPARC** are aggregated and rounded, whereas **antisense** (AS) lines that express low levels of the protein ae flat and spread. We tested whether the cell lines would exhibit these altered morphologies in Ca-2+-deficient media. When cultured under these conditions, S lines attached and spread, whereas AS lines attached but remained round, with no subsequent spreading. Addition of CaCl-2 or purified **SPARC** to the Ca-2+-deficient medium resulted in spreading of the AS and control lines and a reappearance of the altered morphologies. Expression of the Ca-2+-binding cadherin uvomorulin by the cell lines correlated with neither their morphology nor their level of **SPARC** expression. We conclude that the altered phenotypes of the transected lines reflect, in part, the concentration of extracellular Ca-2+ and that the spreading exhibited by the S lines under Ca-2+-deficient conditions is directly related to their enhanced expression of **SPARC**. **SPARC** might, therefore, mediate interactions between cells and matrix that are permissive for adhesion when levels of extracellular Ca-2+ are diminished.

1992

3/3,AB/7 (Item 2 from file: 5)
DIALOG(R)File 5:Biosis Previews(R)

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? s human and osteonectin?
         12599271  HUMAN
             1484  OSTEONECTIN?
      S3      761  HUMAN AND OSTEONECTIN?
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          1961549  INHIBIT?
      S4      127  S3 AND INHIBIT?
? s s4 and py<1996
Processing
              127  S4
         18891041  PY<1996
      S5       50  S4 AND PY<1996
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...examined 50 records  (50)
...completed examining records
      S6       37  RD (unique items)
? t s6/3,ab/all
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6/3,AB/1   (Item 1 from file: 155)
DIALOG(R)File 155:MEDLINE(R)

09454592   94176562   PMID: 8130279

Osteonectin/SPARC is a product of articular chondrocytes/cartilage and is regulated by cytokines and growth factors.

Chandrasekhar S; Harvey AK; Johnson MG; Becker GW

Lilly Research Laboratories, Lilly Corporate Center, Indianapolis, IN 46285.

Biochimica et biophysica acta (NETHERLANDS)   Mar 10 **1994**, 1221 (1) p7-14, ISSN 0006-3002   Journal Code: A0W

Languages: ENGLISH

Document type: Journal Article

Record type: Completed

Rabbit articular chondrocytes maintained in monolayer, synthesized and secreted a 46 kDa protein into the culture medium. N-terminal sequence analysis and immunoprecipitation of the radiolabeled material revealed this protein to be **osteonectin** (ON)/SPARC, a protein previously shown to be present in bone. When chondrocytes were exposed to interleukin-1, a cytokine with matrix degradative properties, ON synthesis and secretion was greatly **inhibited**. However, this was specific to IL-1 since two other pro-inflammatory cytokines (tumor-necrosis factor-alpha and interleukin-6) with properties similar to IL-1, failed to cause any discernible effect on ON synthesis. Several growth factors (TGF-beta, PDGF, and IGF-1), that have been shown to stimulate other cartilage matrix macromolecular synthesis, also stimulated ON synthesis and were also able to reverse the **inhibitory** effect of IL-1 on ON synthesis. These observations were also demonstrated in explant cultures of cartilage. Our studies suggest that ON is a biosynthetic product of articular cartilage and could play a role in cartilage structure and/or function.

6/3,AB/2   (Item 2 from file: 155)
DIALOG(R)File 155:MEDLINE(R)

08803232   96094759   PMID: 7495300

Immunolocalization of SPARC, tenascin, and thrombospondin in pulmonary fibrosis.

Kuhn C; Mason RJ

Department of Pathology, Brown University, Pawtucket, Rhode Island, USA.

American journal of pathology (UNITED STATES)   Dec **1995**, 147 (6) p1759-69, ISSN 0002-9440   Journal Code: 3RS

Contract/Grant No.: HL 27353, HL, NHLBI
Languages: ENGLISH
Document type: Journal Article
Record type: Completed

Several biochemically unrelated multifunctional extracellular proteins, SPARC, thrombospondin 1, and tenascin-C (TN), have been grouped as antiadhesive glycoproteins because they **inhibit** the spreading of cells on extracellular matrix in vitro. Migration of fibroblasts and epithelial cells into the air spaces to organize inflammatory exudate is a feature common to several fibrosing lung diseases. We hypothesized that migration would be facilitated by loosening the adhesive interactions between cells and the pericellular matrix components of the alveolar wall and that one or more of the anti-adhesive glycoproteins could be involved. Immunohistochemistry was used to localize SPARC, TN, and thrombospondin 1 in biopsies of organizing pneumonia, idiopathic pulmonary fibrosis (nine cases of usual interstitial pneumonia, one of desquamative interstitial pneumonia), and control lungs. Each antigen had a distinctive distribution. Only TN was expressed in control lungs, where it strongly stained the basement membrane of large bronchi and weakly stained alveolar entrance rings and small veins. In organizing pneumonia, TN was heavily stained through the entire extracellular matrix of the Masson bodies. In idiopathic pulmonary fibrosis, TN was abundant in the fibroblast foci of active fibrosis but was also present in the basement membrane regions beneath the metaplastic epithelium lining honeycomb cysts. TN was abundant in the interstitium in desquamative interstitial pneumonia. SPARC was observed only intracellularly where it occurred in the fibroblasts of Masson bodies of organizing pneumonia and the fibroblast foci of usual interstitial pneumonia. In desquamative interstitial pneumonia, expression of SPARC was minimal, in rare interstitial fibroblasts. Thrombospondin 1 was found consistently in organizing pneumonia but only infrequently in idiopathic pulmonary fibrosis. In both, it was localized in the extracellular matrix immediately beneath reparative epithelium. These results are consistent with a role for SPARC in fibroblast migration. TN may function in both fibroblast migration and the adhesion of metaplastic bronchial-type epithelium. However, these proteins also have other activities that may be important in pulmonary fibrosis. The localization of thrombospondin 1 suggests that it may be synthesized by regenerating epithelium where it may aid in the adhesion or migration of the epithelial cells.

6/3,AB/3 (Item 3 from file: 155)
DIALOG(R)File 155:MEDLINE(R)

08623698 96032764 PMID: 7559469

Role of N-linked glycosylation in **human osteonectin**. Effect of carbohydrate removal by N-glycanase and site-directed mutagenesis on structure and binding of type V collagen.

Xie RL; Long GL
Department of Biochemistry, School of Medicine, University of Vermont, Burlington 05405, USA.

Journal of biological chemistry (UNITED STATES) Sep 29 **1995**, 270 (39) p23212-7, ISSN 0021-9258 Journal Code: HIV
Contract/Grant No.: CO6-HL39475, CO, NCI; PO1-AG08777, AG, NIA
Languages: ENGLISH
Document type: Journal Article
Record type: Completed

In this study we demonstrate that the binding region of recombinant truncated **human** bone **osteonectin** (tHON) for type V collagen resides between amino acids 1 and 146. After removal of oligosaccharide chain structures from tHON, bovine bone **osteonectin** (BBON) and **human** platelet **osteonectin** (HPON) by N-glycanase, their ability to bind to type V collagen is increased, and HPON affinity to collagen V is the same as that of BBON. These data suggest that glycosylation of **osteonectin** has a direct or regulatory effect on **osteonectin**

binding to collagen V and that the increase in tHON binding upon removal of carbohydrate is the result of a loss of a down-regulation site or direct interference of the carbohydrate at the binding site. To determine the specific role of each N-glycosylation site in tHON, Asn71 and Asn99 were mutated to Gln (N71Q, N99Q) and Thr73 and Thr101 mutated to Ala (T73A, T101A) to selectively **inhibit** oligosaccharide attachment. The binding affinity of N99Q and T101Q to collagen V is markedly increased over wild-type tHON, whereas N71Q and T73A are the same as wild-type tHON. The doubled mutant (N71,99Q) binds identically to collagen V as N99Q and T101A. These data suggest that only the position 99 glycosylation site (Asn99-X-Thr101) in tHON is important in the reduction of binding of **osteonectin** to collagen V. Consistent with the binding data is the observation that both the N71Q and T73A mutant proteins migrate on SDS-polyacrylamide gel electrophoresis gels identically to wild-type tHON, suggesting that there is little or no N-glycosylation of residue 71 in wild-type **osteonectin**.

6/3,AB/4 (Item 4 from file: 155)
DIALOG(R)File 155:MEDLINE(R)

08591784 95377536 PMID: 7649376
Effects of basic fibroblast growth factor on proliferation, the expression of **osteonectin** (SPARC) and alkaline phosphatase, and calcification in cultures of **human** pulp cells.
Shiba H; Nakamura S; Shirakawa M; Nakanishi K; Okamoto H; Satakeda H; Noshiro M; Kamihagi K; Katayama M; Kato Y
Department of Endodontology and Periodontology, Hiroshima University School of Dentistry, Japan.
Developmental biology (UNITED STATES) Aug **1995**, 170 (2) p457-66, ISSN 0012-1606 Journal Code: E7T
Languages: ENGLISH
Document type: Journal Article
Record type: Completed
Basic fibroblast growth factor (bFGF) may be involved in the development and repair of dentine and pulp because bFGF, its related peptides, and FGF receptors are expressed in dental mesenchymal cells. In this study, we examined the effects of bFGF on DNA synthesis, **osteonectin**/SPARC levels, alkaline phosphatase (ALPase) activity, their mRNA levels, and calcium levels in cultures of **human** pulp cells. Pulp cells were isolated from three healthy upper wisdom teeth of three patients and maintained separately. These cells produced SPARC, ALPase, and calcified nodules and there was a close correlation between the SPARC-synthetic activity of the cell lines and their levels of ALPase and calcification. The levels of SPARC, ALPase and calcium deposits in the three pulp cell cultures were 10-250 times those of **human** foreskin fibroblasts. Western blots showed that the pulp cells produced 38-kDa SPARC. Northern blots showed that the pulp cells expressed flg (FGF receptor type 1) transcripts throughout all culture stages, irrespective of the presence or absence of bFGF. The addition of bFGF to the pulp cultures suppressed the increases in ALPase activity, SPARC synthesis, and their mRNA levels, although it increased the incorporation of [3H]thymidine into DNA > 10-fold. The effects of bFGF on ALPase activity and SPARC synthesis were reversible. Furthermore, bFGF abolished the calcification of the extracellular matrix; the calcium content of bFGF-free cultures. These findings suggest that bFGF is a potent mitogen for **human** pulp cells and that it **inhibits** the expression of the odontoblast phenotype by the cells at least partly at pretranslational levels.

6/3,AB/5 (Item 5 from file: 155)
DIALOG(R)File 155:MEDLINE(R)

08444436 95074005 PMID: 7982919

Rol del gen **SPARC** en la capacidad tumorigenica de celulas de melanoma humano.

Ledda MF; Adris S; Bover L; Bravo AL; Mordoh J; Podhajcer OL
Instituto de Investigaciones Bioquimicas Luis Leloir, Fundacion Campoma, Buenos Aires, Argentina.

Medicina (ARGENTINA) 1996, 56 (1) p51-4, ISSN 0025-7680
Journal Code: MMM
Languages: SPANISH
Document type: Journal Article
Record type: Completed

Previous studies from our laboratory have demonstrated that human melanoma cell lines and tumors expressed high levels of the extracellular protein **SPARC** . In order to demonstrate its role in human melanoma progression, IIB-MEL-LES human melanoma cells were transfected with **SPARC** full length c-DNA in the **antisense** orientation. In vivo studies demonstrated that all the control mice injected with parental cells developed tumors, while none of the mice injected with cells obtained from three different clones with diminished levels of **SPARC** expression, developed tumors. These studies suggest that **SPARC** may play a key role in human melanoma progression.

2/3,AB/5 (Item 5 from file: 155)
DIALOG(R)File 155:MEDLINE(R)

07721153 93228971 PMID: 1299273

Overexpression of **SPARC** in stably transfected F9 cells mediates attachment and spreading in Ca(2+)-deficient medium.

Everitt EA; Sage EH
Department of Biological Structure, School of Medicine, University of Washington, Seattle 98195.

Biochemistry and cell biology (CANADA) Dec 1992, 70 (12) p1368-79, ISSN 0829-8211 Journal Code: ALR
Contract/Grant No.: 5T32-GM07270, GM, NIGMS; GM-40711, GM, NIGMS
Languages: ENGLISH
Document type: Journal Article
Record type: Completed

The Ca(2+)-binding protein **SPARC** is one of a group of proteins that function in vitro to promote the rounding of cells. To assess whether the modulation of cell shape by **SPARC** is affected by extracellular Ca2+, we used F9 cell lines that had been stably transfected with sense or **antisense** **SPARC** DNA. Sense-transfected (S) lines that overexpress **SPARC** are aggregated and rounded, whereas **antisense** (AS) lines that express low levels of the protein are flat and spread. We tested whether the cell lines would exhibit these altered morphologies in Ca(2+)-deficient media. When cultured under these conditions, S lines attached and spread, whereas AS lines attached but remained round, with no subsequent spreading. Addition of CaCl2 or purified **SPARC** to the Ca(2+)-deficient medium resulted in spreading of the AS and control lines and a reappearance of the altered morphologies. Expression of the Ca(2+)-binding cadherin uvomorulin by the cell lines correlated with neither their morphology nor their level of **SPARC** expression. We conclude that the altered phenotypes of the transected lines reflect, in part, the concentration of extracellular Ca2+ and that the spreading exhibited by the S lines under Ca(2+)-deficient conditions is directly related to their enhanced expression of **SPARC**. **SPARC** might, therefore, mediate interactions between cells and matrix that are permissive for adhesion when levels of extracellular Ca2+ are diminished.

2/3,AB/6 (Item 1 from file: 5)
DIALOG(R)File 5:Biosis Previews(R)

08087425 BIOSIS NO.: 000093097496

EXPRESSION OF **SPARC** IS CORRELATED WITH ALTERED MORPHOLOGIES IN
TRANSFECTED F9 EMBRYONAL CARCINOMA CELLS

AUTHOR: EVERITT E A; SAGE E H
AUTHOR ADDRESS: DEP. BIOL. STRUCTURE, SM-20, UNIV. WASH., SEATTLE, WASH. 98195.

JOURNAL: EXP CELL RES 199 (1). 1992. 134-146. **1992**
FULL JOURNAL NAME: Experimental Cell Research
CODEN: ECREA
RECORD TYPE: Abstract
LANGUAGE: ENGLISH

ABSTRACT: **SPARC** (secreted protein, acidic and rich in cysteine) is a Ca^{2+}-binding glycoprotein that has recently been identified as a member of a group of proteins that exert antispreading effects on various cultured cells. In addition, **SPARC** is induced during the later stages of F9 stem cell differentiation to parietal endoderm (PE). When treated with retinoic acid and dibutyryl cAMP, F9 cells differentiate into PE and **SPARC** mRNA is increased approximately 20-fold. To determine whether the chronic overexpression or inhibition of expression of **SPARC** would affect the morphology, attachment, or differentiation of F9 cells, were transfected undifferentiated F9 cells with cDNA encoding **SPARC** or **antisense** **SPARC** and cloned lines that expressed either elevated or reduced levels of **SPARC** protein. The transfected F9 cells displayed altered morphologies in culture: cells of four overexpressing lines appeared clumped and rounded, whereas those of three underexpressing lines were spread and flat, in comparison to controls. Moreover, the morphological differences persisted during differentiation of the lines to PE. The altered morphology was not due to an increased expression of collagenases and did not affect the ability of the cells to attach and adhere to tissue culture plastic. The altered phenotype of the transfected F9 cells appeared to be directly related to the level of extracellular **SPARC**. Since overexpression of **SPARC** induced rounding and aggregation of F9 cells in culture, we propose that **SPARC** facilitates modulation of cell-cell or cell-substrate interactions in vivo.

1992

08087425 BIOSIS NO.: 000093097498

EXPRESSION OF **SPARC** IS CORRELATED WITH ALTERED MORPHOLOGIES IN TRANSFECTED F9 EMBRYONAL CARCINOMA CELLS

AUTHOR: EVERITT E A; SAGE E H
AUTHOR ADDRESS: DEP. BIOL. STRUCTURE, SM-20, UNIV. WASH., SEATTLE, WASH. 98195.

JOURNAL: EXP CELL RES 199 (1). 1992. 134-146. 1992
FULL JOURNAL NAME: Experimental Cell Research
CODEN: ECREA
RECORD TYPE: Abstract
LANGUAGE: ENGLISH

ABSTRACT: **SPARC** (secreted protein, acidic and rich in cysteine) is a Ca2+-binding glycoprotein that has recently been identified as a member of a group of proteins that exert antispreading effects on various cultured cells. In addition, **SPARC** is induced during the later stages of F9 stem cell differentiation to parietal endoderm (PE). When treated with retinoic acid and dibutyryl cAMP, F9 cells differentiate into PE and **SPARC** mRNA is increased approximately 20-fold. To determine whether the chronic overexpression or inhibition of expression of **SPARC** would affect the morphology, attachment, or differentiation of F9 cells, were transfected undifferentiated F9 cells with cDNA encoding **SPARC** or **antisense SPARC** and cloned lines that expressed either elevated or reduced levels of **SPARC** protein. The transfected F9 cells displayed altered morphologies in culture: cells of four overexpressing lines appeared clumped and rounded, whereas those of three underexpressing lines were spread and flat, in comparison to controls. Moreover, the morphological differences persisted during differentiation of the lines to PE. The altered morphology was not due to an increased expression of collagenases and did not affect the ability of the cells to attach and adhere to tissue culture plastic. The altered phenotype of the transfected F9 cells appeared to be directly related to the level of extracellular **SPARC**. Since overexpression of **SPARC** induced rounding and aggregation of F9 cells in culture, we propose that **SPARC** facilitates modulation of cell-cell or cell-substrate interactions in vivo.

STIC-ILL

From: Schmidt, Mary
Sent: Monday, March 03, 2003 4:50 PM
To: STIC-ILL
Subject: sparc treatment references

please locate the following references:

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Review

Genes involved in breast cancer metastasis to bone

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Abstract. Metastasis to bone occurs frequently in advanced breast cancer and is accompanied by debilitating skeletal complications. Current treatments are palliative and new therapies that specifically prevent the spread of breast cancer to bone are urgently required. While our understanding of interactions between breast cancer cells and bone cells has greatly improved, we still know little about the molecular determinants that regulate specific homing of breast cancer cells to the bone. In this review, we focus on genes that have been implicated in migration and adhesion of breast cancer cells to bone, as well as genes that promote tumor cell proliferation in the bone microenvironment. In addition, the review discusses new technologies, including better animal models, that will further assist with the identification of the molecular determinants of bone metastasis and will guide the development of new therapies.

Key words. Breast cancer; metastasis; bone resorption; gene; new therapy.

The clinical problem

Metastasis to bone is a common and painful consequence of advanced breast cancer. Bone is the most prevalent site of first distant relapse of breast cancer, with approximately 70% of patients with advanced breast cancer suffering from bone metastases [1, 2]. The majority of bone metastases from breast cancer are osteolytic. Resorption of bone in these metastases leads to complications including osteoporosis, hypercalcemia, spinal cord compression and fractures of the long bones. Quality of life becomes an issue for these patients, especially since the median survival after diagnosis of bone disease is considerably longer (20–30 months) than in patients who are first diagnosed with soft tissue metastases (3–5 months) [2]. The morbidity associated with bone metastasis and the long clinical course of the disease in patients with bone complications highlight the need for effective therapies.

Once bone disease has developed, current therapies are rarely curative. Palliative radiotherapy and surgery are occasionally used to reduce the size of tumor deposits in bone. Chemotherapy is usually not successful, due to drug resistance that commonly develops during progression of the disease. Bisphosphonates have been used for the past two decades to treat hypercalcemia and skeletal metastases. These drugs reduce the frequency and severity of skeletal complications by preventing bone resorption, although survival is generally not improved in patients with advanced breast cancer [3, 4]. Bisphosphonates incorporate into the bone matrix, rendering it more resistant to resorption by osteoclasts. In addition, they inhibit the formation and osteolytic activity of osteoclasts and induce apoptosis in osteoclasts [5, 6]. Bisphosphonates also exert pro-apoptotic and anti-adhesive effects on tumor cells in vitro; however, the importance of these effects in vivo has yet to be determined [reviewed in ref. 7].

New treatments that specifically prevent or reduce metastasis of breast cancer to bone are urgently required. Development of such therapies requires a better understand-

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ing of the molecular mechanisms responsible for the specific homing and proliferation of breast cancer cells in bone. However, research in this field has been hampered by a lack of physiologically relevant models. Most studies to date have made use of the cell line MDA-MB-231 that was derived from a pleural effusion in a breast cancer patient [8]. MDA-MB-231 cells form lung metastases after intravenous injection. After injection into the left ventricle of the heart, MDA-MB-231 cells proliferate in bone, but rarely form non-osseous metastases [9, 10]. The MDA-MB-231 intracardiac model has been informative for investigating the later stages of metastasis, including proliferation of breast cancer cells in bone. However, the poor rate of metastasis to non-osseous sites has made it difficult to verify the specificity of the effects of genes on metastasis to bone. An orthotopic model of breast cancer metastasis to bone and soft tissue sites that has been developed will greatly facilitate research in this field [11]. In this syngeneic Balb/c mouse model, bone and soft tissue metastases arise spontaneously after mammary fat pad injection of the tumor cell line 4T1.2. Independent cell lines derived from the same primary tumour as 4T1.2 exhibit different metastatic phenotypes making this a powerful model for investigating site-specific metastasis.

The ability to metastasize is the single most important life-threatening characteristic of a malignant breast tumor. Metastasis is a multistage process that begins with the acquisition of a motile and invasive phenotype by primary tumor cells. Intravasation of local capillaries and lymph ducts is followed by circulation around the body and arrest by adhesion to the vessel wall. Extravasation from the vessel allows infiltration and proliferation in specific secondary tissues [12].

Why is bone such an attractive site for breast cancer cells? Bone is a highly vascularized organ that provides a fertile environment for colonization by tumor cells. However, it has long been recognized that metastasis is not a random process and that different types of tumors preferentially metastasize to specific sites. The principle of specific interaction between breast cancer cells and the bone microenvironment was first postulated by Paget in 1889. After studying autopsy records of 735 breast cancer patients, he recognized that 'in cancer of the breast the bones suffer in a special way, which cannot be explained by any theory of embolism alone' [13]. Other tumor types, including renal, lung, thyroid and prostate cancer, also demonstrate a predilection for growth in bone [14], while some, including stomach and ovarian cancer, rarely metastasize to bone. Thus, cells of different lineages differ in their ability to home to bone, to proliferate in the bone environment and to interact with stromal components of bone.

This review will consider genes that are involved in the steps leading to bone metastasis. Table 1 lists genes that are important in breast cancer metastasis. Genes highlighted in bold have been implicated in bone metastasis and will be discussed here. The first part of the review focuses on genes that have been implicated in homing of breast cancer cells to bone. The next part concentrates on genes that are involved in interactions between breast cancer cells and bone cells. In particular, the role of bone resorption, and the possible role of osteoclast-independent osteolysis in the development of bone metastases will be discussed. Finally, the review considers new techniques, including better animal models, which together with array technologies will greatly assist with the identification of the molecular determinants of bone metastasis.

Table 1. Genes involved in breast cancer metastasis.

Stage of metastasis	Gene	Ref.
Chemotaxis	**chemokine receptor CXCR4**	41
	osteonectin	46
Invasion and adhesion	**$\alpha v\beta 3$ integrin**	54
	autocrine motility factor	103
	bone sialoprotein	104
	cathepsin D	105
	cortactin	20
	galectin-3	106
	matrix metalloproteinases	28, 90
	osteopontin	107
	urokinase system	31
	semaphorin	108
Growth in bone	**insulin-like growth factor-1**	109
	interleukin-11	87
	parathyroid hormone-related protein	82, 83
	transforming growth factor-β	66
Metastasis suppressors	breast cancer metastasis suppressor 1 (BRMS1)	110
	E-cadherin	10, 15
	Kai1	111
	KiSS-1	112
	Nm23	113
	tissue inhibitors of metalloproteinases	21

Genes implicated in metastasis to bone are in bold and will be discussed in more detail in the review. The RefSeq/LocusLink designation for these genes is available from http://www.ncbi.nlm.nih.gov/LocusLink.

Homing determinants

Breast cancer cells express a number of genes that may act as homing determinants to facilitate their migration to bone. These include genes that promote invasion and allow extravasation from capillaries within bone marrow, genes that confer responsiveness to chemotactic cues and genes that allow adhesion to the bone extracellular matrix.

Invasion and motility

Acquisition of an invasive phenotype is an essential step in metastasis. Metastatic cells lose expression of genes such as E-cadherin that mediate adhesive interactions at the primary site and hinder extravasation from the vasculature [15]. In contrast, metastatic cells gain expression of integrins that enhance adhesive interactions at the secondary site. Metastatic cells become motile by modifying their cytoskeleton to form invadopodia and lamellipodia by expression of genes such as cortactin [16, 17]. In addition, secretion of proteases including matrix metalloproteinases (MMPs) and urokinase plasminogen activator (uPA) allows degradation of the basement membrane and connective tissue, and access to the secondary site [18, 19].

Each of the genes described above has been implicated in metastasis to bone using the MDA-MB-231 intracardiac model. Thus, overexpression of cortactin in MDA-MB-231 cells has been shown to promote osteolytic lesions, but not metastasis to other sites, after intracardiac injection in nude mice [20]. In contrast, expression of a dominant negative cortactin mutant decreased skeletal tumor burden in this model. Similarly, restoring E-cadherin expression, overexpressing the MMP inhibitor TIMP-2, or disrupting the function of the UPA receptor (uPAR) in MDA-MB-231 cells all reduce the frequency and size of osteolytic lesions and diminish skeletal tumor burden in nude mice after intracardiac injection compared to parental MDA-MB-231 cells [10, 21, 22]. However, the studies do not demonstrate that these genes specifically influence metastasis to bone. Since E-cadherin, cortactin, MMPs and uPA are likely to have a general role in metastasis, demonstrating a bone-specific effect using the MDA-MB-231 model that rarely metastasizes to non-osseous tissue is difficult. Further work with the 4T1.2 orthotopic model that spontaneously metastasizes to both bone and soft tissue will be useful to delineate the roles of these genes in metastasis to bone. This will complement other studies, described below, that have investigated the role of these genes in metastasis.

E-cadherin

E-cadherin is a transmembrane protein involved in cell-cell adhesion. Loss of E-cadherin expression has been linked to many types of cancer, including breast cancer where invasive and metastatic cancers express reduced levels of E-cadherin [15, 23]. In addition to reducing skeletal tumor burden, there is some evidence that overexpression of E-cadherin in the MDA-MB-231 intracardiac model also results in decreased metastasis to non-osseous sites [10]. E-cadherin expression does not alter anchorage-dependent growth, suggesting that reduced metastasis is not due to impaired cell growth or decreased tumorigenicity [10]. In this model, E-cadherin-mediated adhesion appears to hinder extravasation of tumor cells from the vascular system to both bone and soft tissue. While this study implicates E-cadherin as an important metastasis suppressor, E-cadherin is unlikely to play a specific role in metastasis to bone.

Cortactin

Gene amplification at the 11q31 chromosomal band and the consequent overexpression of cortactin (EMS1) is frequently associated with breast cancer [24]. Cortactin activates the Arp2/3 complex, a central regulator of actin dynamics [25]. This interaction facilitates actin cytoskeleton reorganization leading to the formation of lamellipodia and membrane ruffles [16]. Anti-cortactin antibodies block matrix degradation at invadopodia in MDA-MB-231 cells [17]. Interestingly, cells overexpressing cortactin demonstrate enhanced adhesion to bone endothelial cells compared with parental cells [20]. These studies underline the importance of cortactin in cell invasion and suggest cortactin may be involved in vascular adhesion in bone metastasis. Further work using new models of bone metastasis will help to elucidate the role of cortactin in bone metastasis.

Matrix metalloproteinases

MMPs comprise a family of over 20 members of zinc-dependent extracellular or membrane-bound proteinases. MMPs function in tissue remodeling and repair by cleaving a range of extracellular matrix proteins [26, 27]. Expression of MMPs by stromal cells is often increased in response to the presence of a tumor or other pathological condition. In addition, tumors can express high levels of one or more MMP family members. MMPs are involved in many stages of tumor progression from expansion of the primary tumor to initiating and maintaining local and distal invasion [19]. Thus, synthetic MMP inhibitors reduce primary tumor growth and local invasion, and decrease skeletal tumor burden in the MDA-MB-231 intracardiac model [28–30]. MMP activity is regulated at a transcriptional level, post-translationally by protease cleavage and by endogenous MMP inhibitors known as tissue inhibitors of metalloproteinases (TIMPs) [27]. MDA-MB-231 cells overexpressing TIMP2 exhibit a decreased ability to invade endothelial cell monolayers, indicating that MMPs are likely to facilitate cell extravasation from the marrow sinus [21] but, again, further work is needed to demonstrate a specific role in metastasis to bone.

The urokinase system

uPA is a secreted serine protease that has various biological roles related to extracellular proteolysis. It converts the inactive zymogen plasminogen into the active serine protease plasmin, which cleaves extracellular matrix components including laminin, fibronectin and collagen [18]. Via interactions with integrins, uPA also regulates

cell adhesion and proliferation [31]. Through these actions, uPA promotes tumor cell migration and invasion. Thus, patients with elevated uPA levels in breast cancer tissue have a poor prognosis [32] and overexpression of uPAR in a rat breast cancer cell line leads to increased primary tumor growth and metastatic progression in vivo [33].

Recently, several studies have addressed the role of the urokinase pathway in the development of osseous breast cancer metastases. The presence of tumor cells expressing uPA in bone marrow has been shown to have prognostic relevance in breast cancer patients, correlating with a significantly shorter metastasis-free interval (36 months) compared to patients who were uPA negative (44.5 months) [34]. However, uPA expression in primary breast tumors appears not to be predictive of metastastic outcome. In a study of 144 patients, there was no significant difference in uPA activity in tumors from patients who were disease free post-operatively, compared with those who went on to develop soft tissue or bone metastases [35]. This suggests that uPA may not be important specifically for invasion to bone but may promote growth of tumor cells that are already in bone. Indeed, uPA is mitogenic for a number of cell types including osteoblasts and prevents apoptosis of MDA-MB-231 cells [36, 37]. These actions of uPA may promote breast cancer progression and contribute to bone metastasis.

Levels of uPA, uPAR and plasminogen activator inhibitor type-1 (PAI-1) have been profiled in human breast carcinomas and their bone metastases, using in situ hybridization [38]. Both uPA and uPAR levels were elevated in malignant cells compared to normal breast epithelium. uPA was expressed in similar amounts in non-invasive and invasive tumors and in bone metastases, whereas uPAR mRNA levels were increased in the latter two tissue types. Thus, uPAR but not uPA appears to correlate with metastatic disease, but not specifically with bone metastases [38].

In addition to mediating proteolytic actions, uPAR is present at the cell surface in functional complexes with integrins where it regulates cell interactions with the extracellular matrix [39]. Exogenous administration or endogenous expression of a peptide that interferes with formation of uPAR/β1 integrin complexes on MDA-MB-231 breast tumor cells inhibits tumor burden in bone following intracardiac injection [22]. This work shows that uPAR/integrin complexes are involved in tumor progression. Further work will clarify whether adhesive and proteolytic events mediated by uPAR signaling play a role in metastasis to bone.

Chemoattraction

In contrast to normal breast epithelial cells, breast tumor cells frequently express proteins that in hemopoietic cells have been shown to function as homing factors [40, 41]. This has led to the hypothesis that these molecules may mediate homing of breast cancer cells to specific sites, including bone.

CXCR4 chemokine receptor

Chemokines are small cytokine-like proteins that elicit directional cell migration and activate signaling pathways that regulate cytoskeletal rearrangement and adhesion [42, 43]. Chemokines are critical for the development and homing of hemopoietic cells to specific organs including bone marrow [43]. There is evidence that similar mechanisms may be involved in homing of breast cancer cells to specific secondary sites. Upregulated expression of the chemokine receptor CXCR4 was recently reported on human breast cancer cell lines, malignant breast tumors and metastases, compared to normal mammary epithelial cells [41]. High levels of mRNA encoding the CXCR4 ligand, CXCL12/SDF-1α, are present in lymph node, lung, liver and bone marrow, which are common sites of breast cancer metastasis. In contrast, organs that are rarely targets of breast cancer metastasis, including kidney and small intestine, express low levels of CXCL12 mRNA.

Consistent with a role in metastasis, the chemokine CXCL12 increases pseudopodia formation, directional migration and invasion in MDA-MB-231 cells, and these events can be blocked by neutralizing anti-CXCR4 antibody [41]. Furthermore, neutralizing anti-CXCR4 antibodies inhibit primary tumor growth in the mammary gland, as well as metastasis to lung and lymph nodes after intravenous injection of MDA-MB-231 cells. Since CXCL12/CXCR4 interactions have been implicated in the homing and repopulation of human stem cells into the bone marrow of SCID mice [44], a similar mechanism may be involved in metastasis of breast cancer cells to bone. However, this remains to be investigated, since the study described above did not use models that are suitable for studying bone metastasis. If expression of CXCR4 by breast cancer cells is shown to mediate homing to bone, this receptor or its ligand may be good targets for future therapies against tumor progression and metastasis. However, examining the consequences of inhibiting CXCR4 on normal physiology, including immune function, will be important.

Osteonectin

Osteonectin (SPARC/BM-40) has been proposed to be a chemoattractive agent that can induce homing of breast cancer cells to bone. Osteonectin is one of the most abundant non-collagenous matrix proteins in bone. It regulates cellular interactions with the extracellular environment by controlling turnover and assembly of the extracellular matrix, and by modulation of growth factor activity and cellular morphology [45]. MDA-MB-231 breast cancer

cells exhibit increased chemotaxis and invasion in vitro in response to osteonectin, but not to other bone-derived proteins including bone morphogenic protein-4 [46]. Treatment with exogenous osteonectin downregulates the MMP inhibitor TIMP2 and stimulates MMP-2 activity in MDA-MB-231 cells, providing a potential link with the enhanced invasive ability of breast cancer cells induced by osteonectin [47].

Whether chemoattraction by osteonectin is responsible for homing of breast cancer cells to bone in vivo remains to be determined. The osteonectin receptor has not been identified. Osteonectin is not expressed in normal mammary tissue or benign breast lesions [48, 49]. Similarly, several breast cancer cell lines, including MDA-MB-231 cells, do not express osteonectin [50] and therefore have the potential to respond to an osteonectin chemoattractive gradient. However, in contrast to MDA-MB-231 cells, both in situ and invasive breast carcinoma lesions show strong expression of osteonectin [49, 50], suggesting that these cells may not be responsive to chemoattraction by osteonectin. While osteonectin may contribute to an invasive phenotype in breast cancer cells through activation of MMPs, further work is required to determine if the presence of osteonectin in bone provides a homing stimulus for breast cancer cells in vivo.

Adhesion

The ability to attach to extracellular matrix molecules within bone is required for breast cancer cells to gain a foothold. Expression of $\alpha v\beta 3$ integrin by breast cancer cells may be important for adhesion within the bone microenvironment.

$\alpha v\beta 3$ integrin

Integrins are cell surface heterodimeric glycoproteins that mediate cellular interactions with the extracellular matrix. $\alpha v\beta 3$ integrin is abundantly expressed by osteoclasts and is important in osteoclast-mediated bone resorption. It is required for migration of osteoclasts and for maintenance of the osteoclast sealing zone during bone resorption [51]. Expression of $\alpha v\beta 3$ integrin has been observed in normal breast epithelial cells, primary human breast tumors, invasive breast cancer lines and bone metastases [52–55]. $\alpha v\beta 3$ integrin binds the tripeptide Arg-Gly-Asp (RGD) that is present in extracellular matrix proteins found in bone, including vitronectin, osteopontin and bone sialoprotein, and mediates binding of breast cancer cells to trabecular bone [56]. In addition, $\alpha v\beta 3$ integrin regulates migration of breast cancer cells and possibly invasion [57–59]. Consequently, a role for $\alpha v\beta 3$ integrin has been postulated in specific homing of breast cancer cells by mediating adhesion and migration of breast cancer cells to the bone extracellular matrix.

$\alpha v\beta 3$ integrin regulates a cell death suppression signal in breast cancer cells. Adhesion to osteopontin through $\alpha v\beta 3$ integrin blocks apoptosis in cells sensitive to phorbol esters [60]. Anti-$\alpha v\beta 3$ antibodies and RGD-containing peptides, but not anti-$\alpha v\beta 5$ antibodies, inhibit survival. By allowing adhesion to bone matrix proteins, $\alpha v\beta 3$ may provide a survival advantage to breast cancer cells that have metastasized to the bone.

Recent work suggests that the activation state of $\alpha v\beta 3$ integrin is an important determinant of metastasis. Overexpression of constitutively active $\alpha v\beta 3$ integrin in the highly metastatic human breast cancer cell line MDA-MB-435 enhances lung metastasis after tailvein injection into SCID mice, compared to cells expressing inactive $\alpha v\beta 3$ or not expressing $\alpha v\beta 3$ [61]. The role of activated $\alpha v\beta 3$ integrin in metastasis to bone has yet to be investigated.

Interactions between breast cancer cells and bone cells

Under normal physiological conditions, bone undergoes constant remodeling. New bone is laid down by stromal cells called osteoblasts. To balance the production of new bone, osteoblasts stimulate the fusion and maturation of osteoclast precursors to generate bone-resorbing osteoclasts. Osteoclasts secrete proteases and acids that dissolve bone matrix and resorb calcified bone. The tightly regulated interaction between osteoblasts and osteoclasts ensures the maintenance of bone integrity.

The recently identified RANKL cytokine signaling system is important in osteoclast-osteoblast interactions. Receptor activator of NF-κB ligand (RANKL) is a member of the tumor necrosis factor (TNF) family of cytokines and is expressed by osteoblasts (fig. 1) [62]. RANKL binds its receptor RANK on osteoclast precursors and induces their maturation into multinucleated bone-resorbing osteoclasts. Interactions between RANKL and RANK are inhibited by the soluble decoy receptor osteoprotegerin (OPG) [63, 64]. OPG is produced by osteoblastic stromal cells and acts locally to neutralize RANKL, thereby inhibiting activation of osteoclasts. Thus, osteoblasts regulate osteoclast activity by production of both activating and inhibitory proteins.

In bone metastasis, interactions between breast cancer cells and bone cells set up what has been termed the vicious cycle, resulting in increased osteoclast-mediated bone resorption [65]. Bone contains a diversity of growth factors including insulin-like growth factors, transforming growth factor-β (TGF-β), bone morphogenic proteins and fibroblast growth factors sequestered in the bone matrix. These are released into the bone microenvironment by osteoclast-mediated bone resorption. TGF-β, one of the most abundant of the bone-derived factors, stimulates

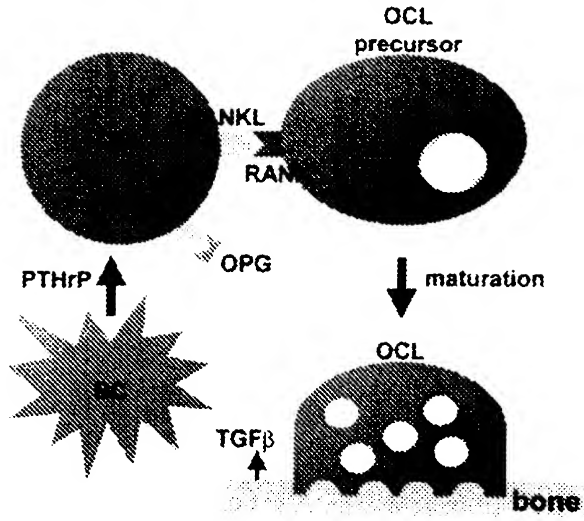

Figure 1. Breast cancer cells interact with bone cells. Osteoblasts (OBL) regulate osteoclast (OCL) maturation and activity by the alternate expression of RANKL, an osteoclast-activating factor, or OPG, an inhibitory decoy receptor. Breast cancer cells (BC) respond to release of bone-derived factors such as transforming growth factor-β (TGFβ) by producing parathyroid hormone-related protein (PTHrP). PTHrP enhances RANKL expression and decreases OPG expression by osteoblasts, leading to enhanced osteoclast activity.

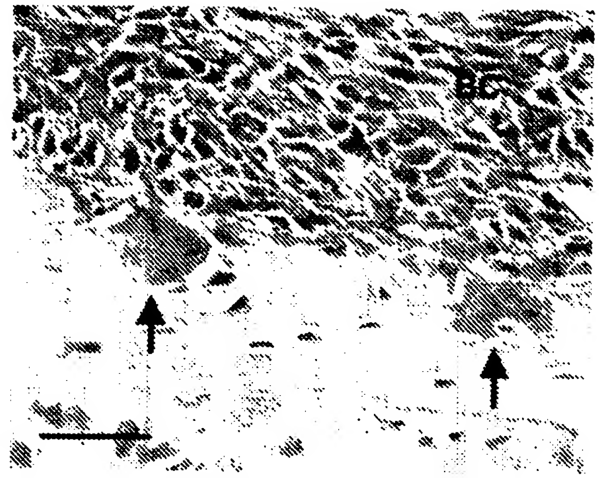

Figure 2 Hematoxylin and eosin-stained section through bone showing breast cancer cells (BC) adjacent to resorbing osteoclasts (arrows). Scale bar: 40 µm. The section is taken from an orthotopic mouse model of breast cancer metastasis

breast cancer cells to produce the potent osteoclast-stimulating factor parathyroid hormone-related protein (PTHrP) [66]. This induces further activation of osteoclasts and increases bone resorption (fig. 1).

Breast cancer cells disturb the balance between osteoblasts and osteoclasts by interfering with RANKL signaling [67]. In co-cultures of osteoclast precursors, osteoblasts and breast cancer cells, overexpression of PTHrP by the tumor cells enhances RANKL mRNA expression and decreases OPG mRNA expression in osteoblasts [67]. This increases production of active osteoclasts, leading to further osteoclast-mediated bone resorption and release of growth factors. Whether breast cancer cells can directly activate osteoclasts is uncertain. In a study of 18 samples, primary breast tumors were shown to express RANK and OPG, but not RANKL [67], suggesting that direct activation is not likely. However, investigation of gene expression profiles of breast cancer cells that are growing in the bone microenvironment will be necessary to conclude whether they can act as surrogate osteoblasts and directly activate osteoclasts.

Bone resorption accompanies osseous metastatic disease and is responsible for the skeletal complications (fig. 2). However, is bone resorption *essential* for proliferation of breast cancer cells and development of bone metastases? Experiments that alter bone resorption in the MDA-MB-231 intracardiac model indicate that bone resorption and the occurrence of metastases are intimately linked. For example, inhibiting osteoclast activation by treatment with recombinant OPG reduces skeletal tumor burden in nude mice after intracardiac injection of MDA-MB-231 cells [68]. Overexpression of PTHrP in the same model increases the number of osteolytic lesions [69]. In contrast, treatment with anti-PTHrP antibodies results in decreased bone resorption due to a reduction in osteoclast numbers and is accompanied by reduced tumor burden in bone [69]. Similarly, inhibiting endogenous PTHrP production by MDA-MB-231 cells by overexpression of a dominant negative TGF-β receptor that inhibits TGF-β signaling reduces tumor burden in the bones of nude mice after intracardiac injection [66, 69]. Restoration of TGF-β signaling increases PTHrP production by the tumor cells and enhances bone metastasis in vivo.

Bone resorption may be important for releasing from the bone matrix cytokines that promote proliferation of breast cancer cells. In particular, insulin-like growth factor-1 (IGF-1) is a likely candidate. It is an abundant protein in the bone matrix and stimulates the proliferation of normal and transformed breast epithelial cells [70].

Overexpression of a dominant negative IGF type I receptor that impairs responsiveness to IGF-1 reduces skeletal tumor burden in the MDA-MB-231 intracardiac model, indicating that the ability to respond to IGF-1 promotes bone metastasis [71]. Other cytokines present in the bone microenvironment, including TGF-β and interleukin-11 (IL-11), are less likely to stimulate growth of tumor cells. IL-11 inhibits proliferation of both breast cancer cell lines and solid tumors [72, 73]. TGF-β inhibits proliferation of primary human mammary epithelial cells and loss of sensitivity to its effects has been associated with tumorigenesis [74, 75].

While bone resorption is consistently observed with bone metastasis and could stimulate tumor cell proliferation

through release of growth factors, a causal role has yet to be shown. Experiments to determine if bone metastases can form in osteopetrotic animal models where bone resorption is defective would provide a more definitive answer. Several osteopetrotic mouse models including mice deficient for $\beta 3$ integrin or RANK may be useful for such experiments [76].

Parathyroid hormone-related protein

PTHrP was identified as the causal agent in humoral hypercalcemia of malignancy [77]. It is a potent osteoclast-stimulating factor that elevates blood and renal calcium levels by promoting osteoclast-mediated bone resorption [78]. PTHrP promotes branching morphogenesis in the developing breast and is produced during lactation [79]. Seventy percent of primary breast tumors express PTHrP [80–82]. In addition, an immunohistochemical study documented PTHrP expression in 12 of 13 breast cancer-derived bone metastases, compared with 3 of 18 metastases to non-osseous sites [83]. These observations implicated PTHrP as important in resorption associated with bone metastasis from breast cancer.

Several retrospective clinical studies show that expression of PTHrP in primary breast tumors correlates with increased bone metastasis, but not recurrence or reduced survival. [81, 84, 85]. Consequently, PTHrP has been suggested as a useful prognostic indicator of metastasis to bone. However, a recent prospective study questions the prognostic significance of PTHrP expression by primary breast tumors [82]. The study of 367 consecutively accrued breast cancer patients showed that PTHrP expression in the primary tumor associated independently with *improved* survival [82]. Consistent with previous studies, PTHrP was detected in 72% of primary tumors. In contrast to previous studies, patients with PTHrP-positive tumors were less likely to develop either bone or soft-tissue metastases than those with PTHrP-negative tumors, suggesting that PTHrP expression in the primary tumor correlates with a less invasive phenotype. Those patients who returned for follow-up bone surgery, however, presented with PTHrP-positive bone metastases, and included some patients who had had PTHrP-negative primary tumors. This study suggests that PTHrP expression is regulated by the bone microenvironment and may facilitate growth of breast cancer cells after they have metastasized to bone. Consistent with this concept, human breast cancer cells isolated from a metastatic bone lesion in an experimental metastasis model express higher levels of PTHrP than the parental population [86]. The finding that PTHrP is expressed by the majority of breast cancer-derived bone metastases, but by only a small proportion of metastases to non-osseous sites [83], is consistent with specific upregulation of PTHrP within the bone microenvironment. The conclusion drawn in that study was that PTHrP expression in the primary tumor favors metastasis to bone; however, PTHrP expression by the primary tumors was not examined to confirm this explanation. Furthermore, experiments using the 4T1.2 spontaneous orthotopic mouse model of breast cancer metastasis to bone [11] indicate that PTHrP expression by the primary tumor is not sufficient to induce metastasis to bone. Elevation of PTHrP expression in a cell line that is metastatic to lung was insufficient to induce metastasis to bone [R. Anderson et al., unpublished results]. While PTHrP is unlikely to be a useful prognostic indicator of metastatic outcome, these studies do support the central role of PTHrP in mediating interactions of breast cancer cells with the bone marrow stroma. Consequently, PTHrP may be an appropriate target for new therapies to reduce tumour-associated bone resorption.

Cytokines

The cytokines IL-6 and IL-11 promote osteolysis by stimulating osteoclast formation. Endogenous expression of IL-6 and IL-11 has been documented in invasive primary breast tumors [87]. No significant association has been found between IL-6 status and occurrence of bone metastasis. However, tumors expressing IL-11 mRNA have a significantly higher rate of bone metastases than IL-11 negative tumors [87]. IL-11 is likely to play an important role in promoting osteolysis at the site of bone disease, and expression in the primary tumor may be a useful predictive factor for the subsequent development of bone metastases.

MMPs – osteoclast-independent osteolysis?

The central role of osteoclast-mediated bone resorption in the development of skeletal metastasis is well documented. However, direct degradation of bone by human breast cancer cells has also been observed in vitro and may be mediated by MMPs [30, 88]. Osteoblasts and osteoclasts produce MMPs that regulate bone homeostasis [89]. Metastatic tumor cells present in bone produce MMPs capable of degrading bone matrix collagen [90]. Interestingly, while TGF-β enhances expression of MMP-1 and -9, and TIMP-1 and -2, by MDA-MB-231 cells in culture, it inhibits MMP expression in normal breast epithelial cells [75]. This suggests a potential mechanism whereby the release of TGF-β from the bone matrix during osteoclast-mediated osteolysis may alter expression of MMPs and their tissue inhibitors in bone-metastasizing cancer cells.

These observations suggest that production of MMPs by breast cancer cells may contribute to osteoclast-independent degradation of the bone matrix or connective tissue. This may be especially relevant in the advanced stages of metastatic disease when growth factors such as TGF-β have been released into the bone microenvironment

through resorption of bone. The development of therapies that inhibit tumor-associated bone degradation will need to address the potential role of MMPs in this process. If breast cancer cells are shown to mediate both osteoclast-dependent and -independent osteolysis, inhibition of both processes will be necessary to block the vicious cycle of bone destruction and tumor cell proliferation.

Development of new therapies

The most promising therapies for osteolytic metastases to date are aimed at inhibiting bone resorption. Direct interruption of the molecular interactions between RANKL and RANK using recombinant OPG has generated promising results in animal models, as described above [68]. In addition, a recent phase I clinical study in breast cancer patients with bone metastases showed that a single dose of a recombinant human OPG construct resulted in decreased urinary N-telopeptide (NTX), a marker of bone resorption, at levels comparable to those achieved by treatment with the bisphosphonate pamidronate [91].

Experiments with animal models have indicated that the inhibitory effect of OPG on tumor cell growth appears to be specific to bone metastases since exogenous OPG has no effect on subcutaneous primary tumor growth of prostate cancer cells [92] or on primary breast tumor growth and lung metastases that occur after growth in the mammary fat pad in mice [Anderson et al., unpublished observations]. Furthermore, the reduction in bone metastasis seen with OPG treatment in the MDA-MB-231 intracardiac model did not result in tumor redistribution to other sites [68], which is an important consideration for site-specific metastasis inhibitors. However, this may be attributed to the limited ability of MDA-MB-231 cells to colonize non-osseous sites. Studies in orthotopic models, such as 4T1.2, that also metastasizes to soft tissue will clarify this point.

Similar OPG levels were found in breast cancer patients with no evidence of bone or non-osseous metastatic disease compared with stage IV patients with bone involvement [93]. Thus, OPG expression in primary tumors is unlikely to be a useful prognostic marker of improved outcome or reduced bone metastasis. However, if OPG can be demonstrated to decrease skeletal tumor burden or improve quality of life in patients, it will become a novel and specific therapy to treat tumor-associated bone resorption.

The central role of PTHrP in bone resorption makes it an attractive candidate for new therapies to treat osteolytic bone disease. Humanized anti-PTHrP antibodies are being investigated [94]. Small-molecule inhibitors of PTHrP that reduce osteolysis and skeletal tumor burden in an experimental model of breast cancer metastasis have been identified and may form the basis for future therapies [95]. Therapies that target IL-11 may also be useful to suppress osteolytic bone disease [96]. These include cyclooxygenase inhibitors that target the prostaglandin E2-dependent mechanism of IL-11-mediated bone resorption.

Inhibition of MMP activity is an attractive clinical strategy due to the central role played by MMPs in tumour progression. However, results from phase III trials have shown little or no clinical efficacy [27]. Future trials may need to target patients with specific MMP expression profiles determined at an early stage in their disease [97]. Trials may also be more successful with the development of more potent inhibitors of MMP function. Antagonists of $\alpha v\beta 3$ integrin are being investigated for use in diseases including osteoporosis [98]. With a better understanding of the role of $\alpha v\beta 3$ integrin in bone metastasis, these drugs may be used to prevent adhesion of breast cancer cells to the bone or to reduce bone resorption by inhibiting osteoclast activity.

The future

Considerable advances have been made in identifying genes that facilitate various steps required for bone-specific metastasis, including homing and adhesion to bone, and genes that create and maintain an environment that supports proliferation of tumor cells in bone. Mouse models of metastasis have been invaluable for the identification of these genes. However, most models of breast cancer metastasis have been inadequate at delineating site-specific metastasis. They lack relevant pathophysiological pathways of metastasis since tumor cells are often injected subcutaneously or into the vascular system. While the MDA-MB-231 intracardiac model has been informative, more definitive results will come from new orthotopic models of breast cancer metastasis to bone such as the 4T1.2 model. Another orthotopic model of metastasis to bone may be developed from observations that bone disease develops in SCID mice 4 weeks after removal of primary mammary gland tumors derived from MDA-MB-435 human breast cancer cells [61]. This model would allow the metastatic process to bone to be investigated in vivo using a human cell line.

Models of spontaneous metastasis to bone will allow all stages of breast cancer metastasis to be investigated, from escape of the primary tumor cells to metastatic proliferation at multiple specific sites that mirror the human disease. In addition to confirming the role of previously identified candidates, the new models of metastasis provide a system for unbiased phenotypic or genetic screening for novel genes that influence bone metastasis. Introduction of cDNA libraries into cell lines used in the mouse metastasis models will allow unbiased identification of genes that alter the metastatic capacity of these

cells. cDNA microarray technology will be useful for identifying new diagnostic or prognostic markers of bone metastasis and targets for future therapies. cDNA microarray screening enables simultaneous evaluation of the expression profiles of thousands of genes. This technology has already been used to identify genes that correlate with specific metastatic phenotypes [99–101]. Informative experiments will include comparison of gene expression profiles of samples with specific metastatic phenotypes (for example, bone metastases versus soft-tissue metastases, or primary breast tumours with different metastatic outcomes) from both human samples and animal metastasis models.

In the same way that breast cancers differ in their genetic determinants of tumorgenicity (for example, mutations in Neu/ErbB2 or BRCA1), the dominant determinants for metastatic growth in bone are also likely to vary (for example, bone resorption stimulated by IL-11 or PTHrP). Most current candidate genes have only been investigated in the MDA-MB-231 intracardiac model. Validating these results in the new models of metastasis to bone and in human material will be important. This will be assisted by the development of tissue microarrays [102], which allow rapid screening of large numbers of human tumor samples on a scale not technically feasible by traditional histological methods.

These technologies will provide invaluable tools for characterizing the molecular basis of metastasis to bone. They will complement the current studies of candidate genes and should reveal the genes that are essential for bone metastasis. The results of this research will pave the way for the development of new therapies that will specifically target the steps involved in breast cancer metastasis to bone.

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SPARC (Secreted Protein Acidic and Rich in Cysteine) Induces Apoptosis in Ovarian Cancer Cells

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Secreted protein acidic and rich in cysteine (SPARC) is an extracellular Ca^{2+}-binding matricellular glycoprotein that associates with cell populations undergoing migration, morphogenesis, and differentiation. Studies on endothelial cells have established that its principal functions *in vitro* are counteradhesion and antiproliferation. The mechanism(s) underlying these antitumor effects is unknown. In this study, we showed that SPARC expression in ovarian cancer cells is inversely correlated with the degree of malignancy. The immunohistochemical data presented here confirmed the importance of diminished SPARC expression in ovarian cancer development. Treating human ovarian surface epithelial cells and ovarian cancer cells with SPARC revealed that as SPARC inhibits the proliferation of both normal and cancer cells, it induces apoptosis only in cancer cells. This observation indicates that down-regulation of SPARC is essential for ovarian carcinogenesis as cancer cells become sensitized to the apoptotic activity of SPARC during malignant transformation. We also showed here the first direct evidence that putative SPARC receptors are present on ovarian epithelial cells. Their levels are higher in human ovarian surface epithelial cells than cancer cells. Binding of SPARC to its receptor is likely to trigger tissue-specific signaling pathways that mediate its tumor suppressing functions. Decrease in ligand-receptor interaction by the down-regulation of SPARC and/or its receptor is essential for ovarian carcinogenesis. ***(Am J Pathol 2001, 159:609–622)***

Ovarian carcinoma is the major cause of death among all gynecological malignancies. It is the seventh most common cancer in women worldwide and is the fourth leading cause of death from cancer among American women following lung, breast, and colorectal cancers.[1] The overall 5-year survival rate is only ~30%.[2] More than 90% of human ovarian cancers are thought to arise from the ovarian surface epithelium, which shares the same developmental origin (coelomic epithelium) with the general pelvic and abdominal peritoneum. Ovarian carcinogenesis is a multistep process involving multiple genetic changes. Although several oncogenes (eg, *c-ErbB1*, *HER-2/neu*, *c-fms*, *K-ras*) and tumor suppressor genes (eg, *BRCA1*, *p53*, *DOC-2*) have been implicated to be involved in ovarian tumor formation,[3] the pathogenic mechanisms by which normal ovarian epithelial cells become malignant remain poorly understood.

SPARC (secreted protein acidic and rich in cysteine), also termed osteonectin, BM-40, and 43K protein, is a calcium-binding matricellular glycoprotein that displays a high degree of interspecies sequence conservation.[4–6] This consistency indicates that SPARC performs a basic and important function in animal tissues. Localization by immunohistochemistry and *in situ* hybridization have demonstrated that SPARC is spatially and temporally regulated during development. It is transiently expressed in derivatives of the three primitive germ layers in mouse embryos.[illegible] High levels of SPARC mRNA and protein have been found in developing bones and teeth, principally osteoblasts, odontoblasts, perichondrial fibroblasts, and differentiating chondrocytes in murine, bovine, and human embryos.[11] SPARC also plays important roles in cell-matrix interactions during tissue remodeling, wound repair, morphogenesis, cellular differentiation, cell migration, and angiogenesis.[12]

In fetal and newborn ovaries, highest SPARC expression has been found in granulosa cell precursors and the early stages of oocytes. In the ovaries of 2-week-old immature female mice, the thecal cells around developing follicles showed the highest levels of SPARC expression, whereas low levels are detected in follicular cells and oocytes. In ovaries of pregnant females, the theca expression is maintained, whereas high levels of SPARC are also seen throughout the corpora lutea.[11–13] As for adult human ovary, SPARC is expressed at high levels in ovarian surface epithelium[14] and in the fibrous stroma associated with ovarian carcinomas.[illegible]

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Although SPARC is associated with the extracellular matrix, it does not support cell attachment in vitro.[18] Exogenous SPARC has been shown to induce cell rounding and to inhibit endothelial cells, smooth muscle cells, and fibroblasts from spreading on collagen-coated surface.[19] SPARC also disrupts focal adhesions and alters the distribution of cytoskeletal elements and permeability of endothelial cells.[20] It has been proposed that SPARC interacts with cell surface protein(s) to facilitate changes in cell shape and cell migration.[19,21] Apart from these counteradhesive activities, SPARC also inhibits proliferation of endothelial cells.[22,23] Recent studies have suggested that the counteradhesive effect of SPARC on endothelial cells is mediated through a tyrosine phosphorylation-dependent pathway, whereas its antiproliferative function is dependent on signal transduction via a G-protein-coupled receptor.[24] Nevertheless, neither the SPARC receptor nor the intracellular signaling events that it triggered have been characterized.

Our earlier study demonstrated that SPARC is down-regulated in ovarian carcinoma cells.[25] Restoring SPARC expression in stable transfectants of ovarian cancer cells leads to reduced growth and tumorigenicity.[14] To investigate the unknown mechanism(s) underlying the antitumor activities of SPARC, we have treated normal ovarian epithelial cells and ovarian cancer cells with exogenous SPARC and studied their growth and induction of apoptosis. We have also examined the presence of putative SPARC receptors on ovarian cell surfaces.

Materials and Methods

Human Ovarian Specimens

A total of 12 cases of benign epithelial ovarian tumors, 16 cases of borderline epithelial ovarian tumors, and 58 cases of malignant ovarian carcinomas were studied. These patients were diagnosed and treated for ovarian tumors at Brigham and Women's Hospital, Boston, MA. Patient's consent was obtained before the collection of surgical materials. All tissues were fixed in 10% buffered formalin and embedded in paraffin blocks. The histopathological diagnosis was confirmed by two gynecological pathologists at Brigham and Women's Hospital and Massachusetts General Hospital. The tumors were classified and graded according to the International Federation of Gynecology and Obstetrics (FIGO, 1987) criteria for ovarian tumors. Among those malignant ovarian carcinomas, 21 cases are grade 1, 13 cases are grade 2, and 24 cases are grade 3. Twenty-five normal ovarian tissue samples obtained from patients with other nonneoplastic gynecological diseases were also included in this study for comparison.

Immunohistochemical Staining of SPARC Protein

SPARC was detected on paraffin-embedded tissue sections by the avidin-biotin peroxidase complex (ABC) method using a rabbit polyclonal antibody, LF-54, which was kindly provided by Dr. Larry W. Fisher at the National Institutes of Health (Bone Research Branch, Bethesda, MD). For comparison, some of the specimens were immunostained using a mouse monoclonal antibody generated against the N-terminal region of SPARC (AON-5031; Haematologic Technologies Inc., Essex Junction, VT). Seven-μm-thick tissue sections on gelatin-coated slides were baked at 60°C for more than 2 hours and were deparaffinized in xylene and rehydrated in graded ethanol. For antigen unmasking, sections were immersed in antigen unmasking solution (Vector Laboratories, Inc., Burlingame, CA) and boiled in microwave oven for 10 minutes. Antigen retrieval was done only for procedures using the monoclonal antibody AON-5031. The tissue sections were then washed in phosphate-buffered saline (PBS) for 15 minutes and the endogenous peroxidase activity was blocked by soaking the sections in 0.3% hydrogen peroxide in methanol for 15 minutes. After washing in PBS for 15 minutes, the nonspecific serum binding sites were blocked by incubating the sections in normal goat or horse serum (1:20, Vector Laboratories). Excess serum was then removed and the tissue sections were incubated overnight with LF-54 at a dilution of 1:200 or AON-5031 at 1 μg/ml. After washing the slides in PBS for 30 minutes, the sections were incubated with biotinylated goat anti-rabbit antibody or horse anti-mouse antibody (1:200) for 45 minutes, followed by a 15-minute washing in PBS. For the negative control, PBS instead of the goat or horse antibody was used in the incubation. The sections were subsequently incubated with ABC reagent [ABC reagent contains reagent A (avidin DH) and reagent B (biotinylated horseradish peroxidase H)] for 30 minutes. After washing the tissue sections with Tris buffer for 15 minutes, color was developed by incubating the sections in peroxidase substrate solution (3,3'-diaminobenzidine tetrahydrochloride) for 30 seconds. Finally, the sections were counterstained with hematoxylin, dehydrated, and mounted. The specificity of staining was confirmed by preabsorbing the antibody with purified SPARC protein (Haematologic Technologies Inc.) at 37°C for 2 hours before applying to the sections (1 μg protein per 1 μg IgG).

Semiquantitative Evaluation of Immunoreactivity

The tissue sections immunostained with anti-SPARC antibody were examined by two independent observers unaware of the clinical data. At least five sections were randomly chosen from each specimen for scoring. The intensity of immunoreactivity was graded by a 12-point weighted score that was computed by multiplying the intensity score with the percentage of positive cells score on a section. The intensity score was denoted by a semiquantitative scale: 1, low; 2, medium; and 3, high-staining intensity. The percentage of positive cells score was estimated by the average number of positive cells out of the total number of epithelial cells seen on a section: 0, no positive cells; 1, 1 to 25%; 2, 26 to 50%; 3, 51 to 75%; and 4, >75% of positive cells. Therefore, the weighted scores that range from 0 to 12 represent both the intensity and the

percentage of immunopositive cells. The data obtained were analyzed by one-tailed one-sample t-test. The staining of a tumor sample is considered as significantly different from that of normal ovaries when $P < 0.05$.

Cell Cultures

The primary human mesothelial (MESO) and ovarian surface epithelial (HOSE) cell cultures were established as previously described.[26] The immortalized HOSE cell line HOSE1-15 was obtained by infecting HOSE cells with a replication-defective retroviral construct, LXSN16E6E7, and positive clones were selected using 0.3 mg/ml G418 for 10 days as described.[26] The ovarian carcinoma cell line SKOV3 was purchased from ATCC (Rockville, MD), and all of the other ovarian cancer cell lines used in this study were either established in our laboratory or obtained elsewhere. They were cultured in Medium 199 and MCDB 105 (1:1) supplemented with 10% fetal bovine serum (GIBCO BRL, Rockville, MD).

SPARC Secretion Assay

Secretion of SPARC into culture medium was studied using a primary culture of normal ovarian epithelial cells (HOSE713), an immortalized ovarian surface epithelial cell line (HOSE1-15), and four ovarian carcinoma cell lines (SKOV3, OVCA420, OVCA429, and DOV13). A total of 5×10^4 cells from each cell line were seeded into separate 25-cm^2 tissue culture flasks containing 5 ml of culture medium. Culture medium alone in a flask was also set up as control. After 4 days, the culture medium from each cell line was collected and the cells were counted. The culture medium (2 ml) from each cell line was loaded in separate Centricon-100 centrifugal concentrators and spun in a Sorvall RC-5B refrigerated centrifuge at 2600 rpm for 30 minutes at room temperature. The filtrate from each vial was then collected and loaded in a Centricon-10 concentrator and spun at 5700 rpm for 1 hour at room temperature. After spinning, the retentate was collected and the protein concentration of each sample was determined by the Micro BCA protein assay kit (Pierce, Rockford, IL). To determine the amount of secreted SPARC for each cell line, we also spiked serum-free culture medium with 1, 5, 10, or 25 μg of purified human platelet SPARC proteins (Hematologic Technologies Inc.) and they were concentrated from the medium the same way as described for the cell lines. Western blotting was performed and the SPARC signal intensity was quantified by densitometric analysis.

Western Blot Analysis

Protein samples (25 μg) were mixed with equal volumes of 2× sodium dodecyl sulfate (SDS) sample buffer (125 mmol/L Tris, 2.2 mol/L glycerol, 1.42 mol/L β-mercaptoethanol, 160 mmol/L SDS, 10 mg/L bromophenol blue, pH 6.8), boiled for 10 minutes, and resolved by SDS-polyacrylamide gel electrophoresis (10%). They were then transferred to polyvinylidene difluoride membrane (NEN, Boston, MA) and incubated overnight at 4°C in TBST (10 mmol/L Tris, pH 7.5, 100 mmol/L NaCl, 0.1% Tween 20) containing 5% nonfat dry milk. After washing with TBST, the membrane was incubated in 5% milk containing 2 μg/ml of the monoclonal anti-SPARC antibody AON-5031 (Hematologic Technologies Inc.) at room temperature for 1 hour. The membrane was then washed six times with TBST, each for 5 minutes, and incubated at room temperature with horseradish peroxidase-conjugated donkey anti-mouse IgG (Amersham, Piscataway, NJ) for 1 hour. After washing with TBST, signals were visualized using SuperSignal West Pico chemiluminescent substrate (Pierce).

Growth Kinetics

Cell proliferation was studied using MTT assays and BrdU incorporation enzyme-linked immunosorbent assay (ELISA) (Roche Molecular Biochemicals, Indianapolis, IN). MTT assay is based on the conversion of MTT (a tetrazolium salt) by viable cells into a violet formazan dye that can be quantified by measuring absorbance at 550 nm. HOSE 1-15 or SKOV3 cells (1×10^4/well) were seeded in a 96-well plate in 0.1 ml of culture medium supplemented with different amounts of SPARC. After 72 hours, MTT-labeling reagent was added to 0.5 mg/ml and the cells were incubated at 37°C for another 4 hours. The cells were then lysed by adding 0.1 ml of solubilization solution (10% SDS in 0.01 mol/L hydrochloric acid). After an overnight incubation at 37°C, absorbance at 550 nm (with a reference wavelength of 655 nm) was determined using a Benchmark microplate reader (Bio-Rad, Hercules, CA).

For BrdU ELISA, SKOV3 cells or HOSE1-15 cells (5×10^3 cells/well) were treated for 96 hours at 37°C with different concentrations of exogenous SPARC in a 96-well plate. After incubation, the cells were labeled at 37°C with the pyrimidine analogue BrdU (10 μmol/L) for 6 hours. DNA synthesis was monitored based on the incorporation of BrdU into DNA, which was detected by immunoassay according to the manufacturer's instructions. Cellular proliferation was determined by measuring absorbance at 370 nm (reference wavelength 490 nm).

Analysis of Apoptosis

Induction of apoptosis in SKOV3 cells and HOSE1-15 cells after exogenous SPARC treatment was examined using *in situ* cell death detection (terminal dUTP nick-end labeling (TUNEL)) assay and cell death detection ELISA (Roche Molecular Biochemicals). SKOV3 cells or HOSE1-15 cells (1×10^4 cells/well) were treated with different concentrations of SPARC (0.5 to 20 μg/ml) for 48 hours at 37°C in 8-well chamber slides or 96-well plates. In the TUNEL assay, SPARC-treated or untreated SKOV3 cells on a chamber slide were fixed with 4% paraformaldehyde at room temperature for 30 minutes, and the cells were permeabilized with 0.1% Triton X-100 (in 0.1% sodium citrate) solution for 3 minutes at 4°C. DNA strand breaks induced by apoptosis were detected by the in-

corporation of fluorescein-labeled nucleotides to free 3'-OH DNA ends using terminal deoxynucleotidyl transferase. The number and the staining intensity of the apoptotic cells indicate the extent of apoptotic induction. SKOV3 cells were also stained with DAPI (0.5 μg/ml) for 5 to 10 minutes at room temperature. The early apoptotic cells and DAPI-stained cells were seen under fluorescence microscope.

Cell death ELISA was performed according to the manufacturer's protocol. Briefly, SPARC-treated or untreated SKOV3 and HOSE 1-15 cells were lysed in 200 μl of lysis buffer at room temperature for 30 minutes. The supernatant collected after centrifugation was transferred to a streptavidin-coated microtiter plate. After 2 hours of incubation at room temperature with the immunoreagent mix, the bound mono- and oligonucleosomes on the microtiter plate were washed three times with incubation buffer. After the washes, 100 μl of substrate solution was added to each well. Absorbance at 405 nm (reference wavelength 490 nm) was determined after 10 to 20 minutes of incubation with shaking at room temperature.

Receptor Binding Assay

To prepare the SPARC-AP fusion protein used in the receptor binding assay, cDNA encoding SPARC was polymerase chain reaction-amplified from a SPARC expression vector[14] using T7 primer and a SPARC-coding region reverse primer that contains a *BglII* restriction site (5'-GAA GAT CTT CCG ATC ACA AGA TCC TTG-3'). The amplified DNA fragment was digested with *HindIII* and *BglII*, which was subsequently inserted in-frame upstream of the coding sequence of a thermostable human placental alkaline phosphatase (AP) in the expression vector pAPtag-2 (GenHunter Corp., Nashville, TN). This SPARC-AP construct was co-transfected with the pTK-Hyg plasmid, which contains the hygromycin B resistance gene, into 293T cells. After hygromycin B selection (0.2 mg/ml), stable transfectants that secrete high levels of SPARC-AP proteins were selected and grown to confluence. After 3 days, the culture medium containing the secreted proteins were collected and filtered. As a negative control, AP proteins were similarly prepared from a 293T/pAPtag-4 stable cell line that secretes AP alone (GenHunter Corp.). The secreted proteins in culture medium were used in the receptor-binding assay described below.

In receptor-binding assays, ovarian cancer cells and HOSE cells were grown to ~80% confluent in 60-mm culture dishes. They were rinsed once with HBHA buffer (Hanks' balanced salt solution with 0.5 mg/ml bovine serum albumin and 20 mmol/L HEPES, pH 7.0) and incubated with SPARC-AP or AP proteins (as negative control) at room temperature for 90 minutes. After six washes with HBHA buffer for 10 minutes, the cells were lysed and the endogenous alkaline-phosphatase activities in the lysates were heat-inactivated at 65°C for 20 minutes. AP activities were determined by using an AP assay reagent containing *p*-nitrophenyl phosphate. Increases in AP activities, and hence ligand-receptor binding were indicated by the increases in absorbance at 405 nm.

Table 1. [illegible]

Ovarian tissues	Sample number	Mean weighted score*	Specimens with positive staining (%)
Normal	25	11.5 ± 1.3	100
Benign	[illegible]	10.8 ± 2.4	100
Borderline	[illegible]	[illegible] ± [illegible]†	[illegible]
Invasive			
Grade I	2[illegible]	3.7 ± 2.0†	[illegible]
Grade II	13	[illegible] ± 1.6†	85
Grade III	2[illegible]	0.9 ± 0.[illegible]†	67

*Weighted scores ranging from 0 to 12 represent both the intensity of the immunostaining and the percentage of positive cells.
†Values are given as means ± SEM. Asterisks behind the values denote $P < 0.05$ as determined by one-tailed t-test. These values are significantly different from the mean value obtained from the staining of normal ovaries.

Results

SPARC Expression is Down-Regulated in Ovarian Cancer

Using Northern and Western blotting, we have previously shown that SPARC is highly expressed in HOSE cells. Its expression is reduced in ovarian cancer cell lines and tissues.[14] Recent studies examining the immunoreactivity of SPARC in ovarian tumor tissues revealed different patterns of SPARC expression.[15-17] To investigate the inconsistent pattern of SPARC deregulation in ovarian cancers, we have performed immunostaining for SPARC protein using a polyclonal SPARC antibody LF-54 on 111 paraffin-embedded ovarian tissue samples. Consistent with our RNA and protein data,[14] strong immunoreactivity with a high mean weighted score of 11.5 ± 1.3 was found in the surface epithelial cells of all of the 25 normal ovaries examined (Table 1). Positive staining appeared as dark brown granules spreading throughout their cytoplasm (Figure 1a). No staining was observed in the stromal cells. Strong immunoreactivity (as indicated by the high mean weighted score (10.8 ± 2.4, Table 1) was also detected in all of the benign tumors studied. The staining pattern is similar to that of the normal ovaries, in which most of the surface epithelial cells were positively stained (Figure 1b).

A reduction of immunoreactivity was seen in the cytoplasm of borderline and invasive serous carcinoma cells. In all of the borderline cases examined, < 50% of the surface epithelial cells were stained positive (Figure 1c). The cytoplasmic staining appeared light brown and the intensity was weaker than that of normal ovaries and benign tumors (compare Figure 1, a, b, and c). Although positive cells were detected in all of the borderline tumors examined, they showed a significantly lower ($P < 0.05$) mean weighted score (5.8 ± 0.5, Table 1). Grade I and grade II invasive ovarian tumors also showed significantly decreased staining with mean weighted scores of 3.7 ±

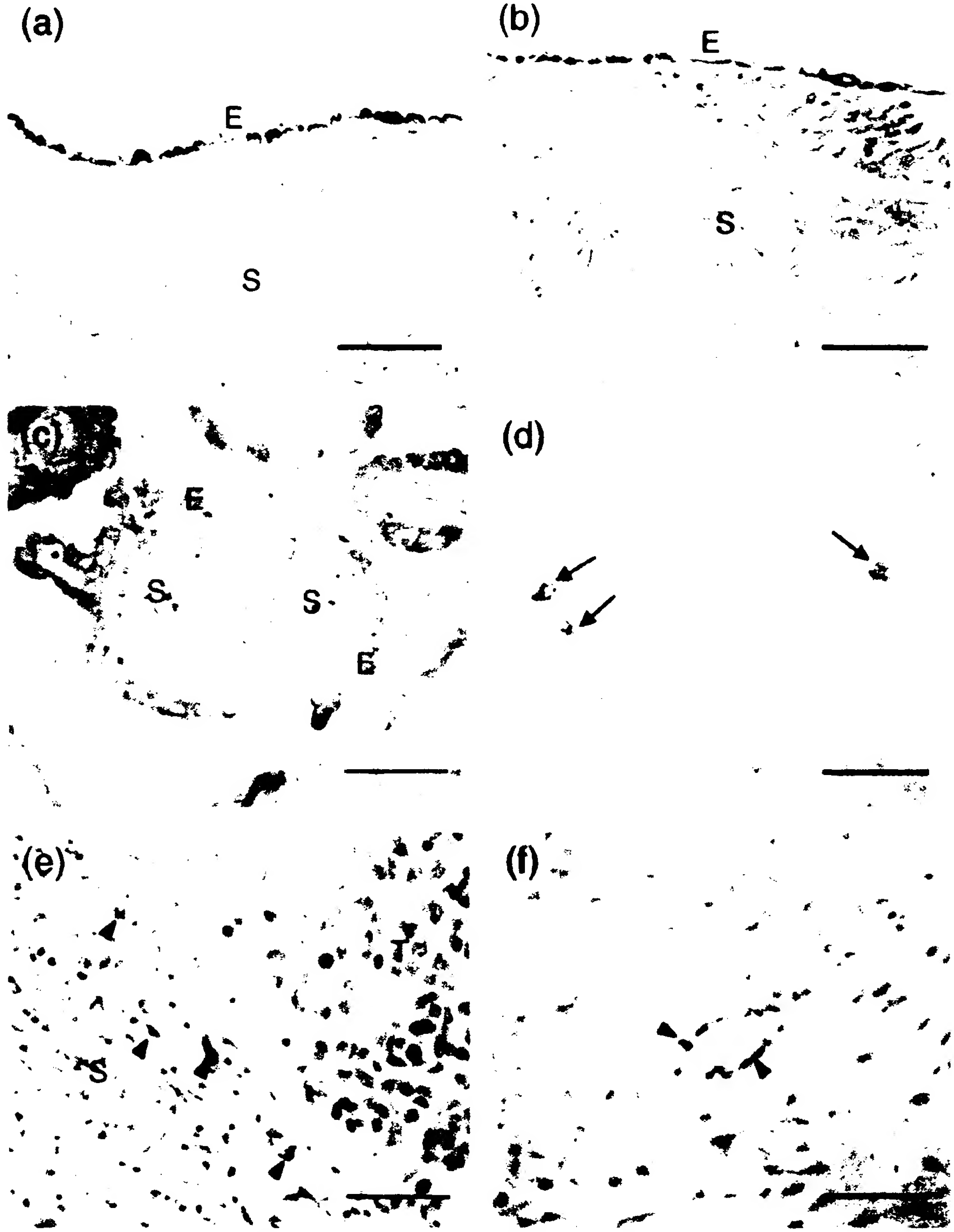

Figure 1. Immunohistochemical analysis and localization of SPARC expression in normal ovary and ovarian tumors. [illegible] (**a**) [illegible] (**b**) [illegible] (**c**) [illegible] (**d**, **e**) and (**f**). All sections were stained [illegible] (**a**, **b**) and (**c**) [illegible] (**d**) [illegible] (**arrows**) (**e**) [illegible] (**arrowheads**) [illegible] (**arrowheads**) (**f**) [illegible]

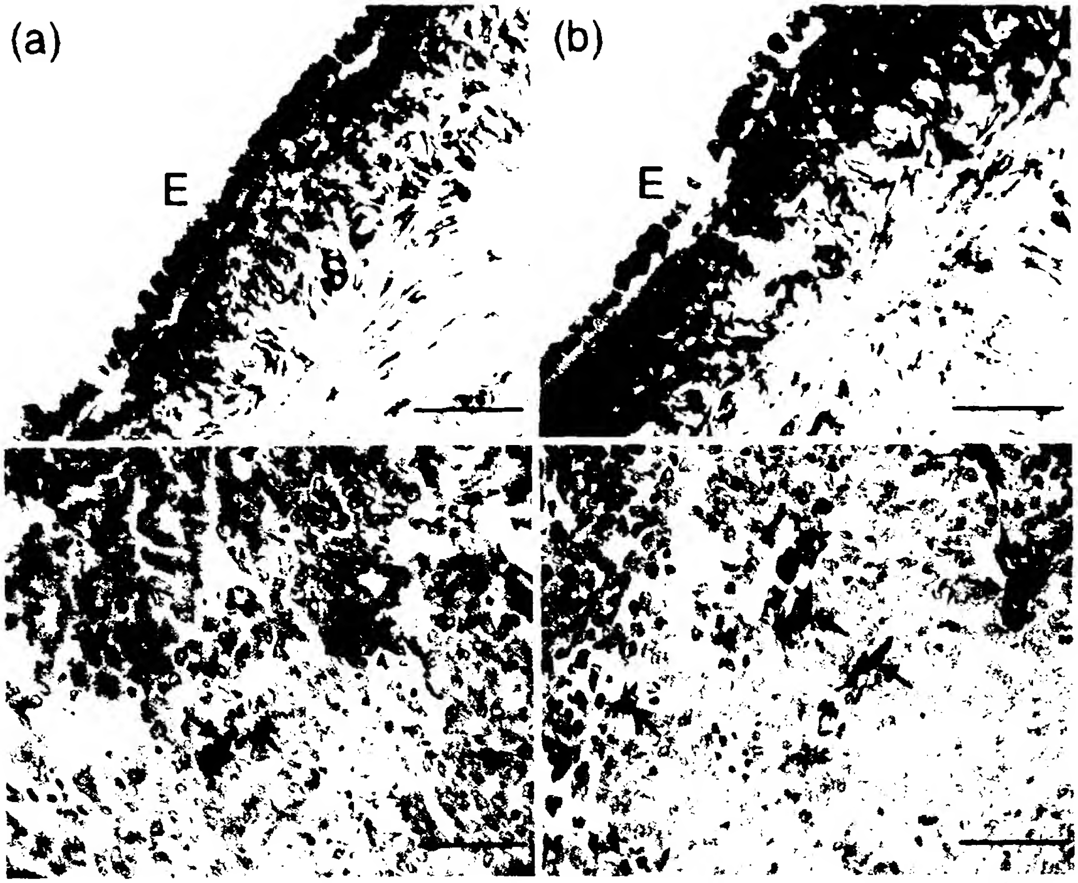

Figure 2. Localization of SPARC immunoreactivity in normal ovary (**a** and **b**) and ovarian cancer (**c** and **d**) using the monoclonal antibody AON-5031. **a:** Very weak staining was observed in the normal ovarian epithelium (E). In contrast, cells in the underlying stroma (S) showed strong SPARC immunoreactivity. **b:** The staining of stromal cells was intensified after heating with antigen-unmasking solution. However, the normal ovarian epithelial cells remain faintly stained. **c:** Positive staining was seen in a few cancer cells and some scattered stromal cells (**arrows**). No immunoreactivity was evident in the vast majority of cancer cells. **d:** Stromal cells (**arrows**) showed strong SPARC staining after antigen retrieval, whereas cancer cells remain unstained. Scale bars, 50 μm (**a** and **b**); 100 μm (**c** and **d**).

2.2 and 1.3 ± 1.6, respectively ($P < 0.05$, Table 1). Positively stained epithelial cells were scattered and only occasionally found (data not shown). Two (15%) of the 13 grade II carcinomas examined did not show any positive epithelial cells. The lowest mean weighted score (0.9 ± 0.1, Table 1) was found in grade III invasive tumors. Light brown cytoplasmic staining was only found in scattered positive epithelial cells (Figure 1d). No stained cells were seen in 13% (3 of 24) of the grade III invasive tumors examined. In addition to the normal and cancerous ovarian epithelial cells, we also observed low levels of immunoreactivity in scattered stromal cells (Figure 1e) and endothelial cells (Figure 1f) of high-grade carcinomas. No signal was seen when the SPARC antibody was incubated with purified blood platelet SPARC protein before applying to the tissue sections, indicating that the immunostainings observed are specific to SPARC.

As different results were reported using the monoclonal antibody AON-5031 for immunohistochemical studies of paraffin-embedded ovarian cancer tissues,[15-17] we have also immunostained some of the paraffin sections to investigate whether similar observation can be obtained using this antibody. Because using high concentrations (>5.4 μg/ml) of AON-5031 for immunostaining have been reported to result in ubiquitous staining throughout the ovary,[17] we used a lower concentration (1 μg/ml) of the antibody in this study. Inconsistent with our results obtained from Northern blotting, Western blotting, and immunostaining, not much positive staining was detected in the surface epithelial cells. However, we found strong immunoreactivity in the stroma underlying the surface epithelium of normal ovary (Figure 2a). On antigen retrieval, the stroma signal was intensified, but the HOSE cells were still faintly stained (Figure 2b). As for ovarian cancer samples, strong immunoreactivity was seen in scattered stromal cells. Although a few epithelial cells were occasionally stained, no immunostaining was evident in the vast majority of cancer cells (Figure 2, c and d). No difference in staining pattern was found in the cancer tissue sections with or without antigen unmasking. We also did not find noticeable difference in staining when 0.5 μg/ml of the antibody was used.

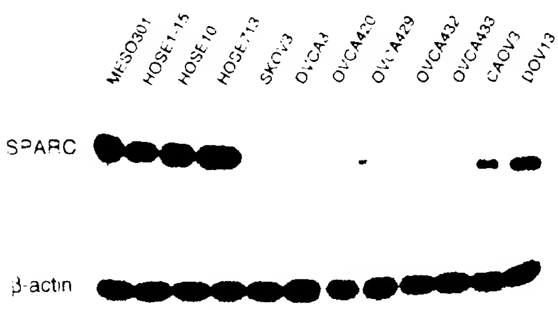

Figure 3. Western blot analysis of total protein lysates prepared from one mesothelial cell line (MESO301), one immortalized HOSE cell line (HOSE1-15), two primary HOSE cell cultures (HOSE10 and HOSE713), and eight ovarian cancer cell lines. The protein lysates (25 μg) were resolved on 10% SDS polyacrylamide gel, transferred to polyvinylidene difluoride membrane, and analyzed with the monoclonal anti-SPARC antibody AON-5031. Detection of β-actin signal at the **bottom** served as a control of the quality of the protein lysates.

Decreased SPARC Secretion in Ovarian Cancers

Because SPARC is a secreted protein, it may affect ovarian cancer growth in an autocrine and/or paracrine manner. Before we analyzed the levels of SPARC secretion in normal and malignant ovarian cell lines, we first confirmed our earlier findings by Western blot analysis showing that SPARC expression is greatly reduced in ovarian cancer. As normal mesothelial and HOSE cells express high levels of SPARC, it was not detected in the cell lysates of most of the cancer cell lines. Among them, DOV13 has the highest SPARC expression level, followed by CAOV3 and OVCA429 (Figure 3). Based on the expression pattern, we anticipate that SPARC is secreted at higher levels in normal ovarian epithelial cells than in ovarian cancer cells. Confirming and estimating the levels of SPARC secretion by normal and malignant ovarian cells are important for validating our subsequent experiments in which the cells were treated with exogenous SPARC.

Culture media from separate cultures of HOSE cells or ovarian cancer cells were collected and concentrated. Equivalent amounts of proteins were run on polyacrylamide gels and analyzed by Western blotting. We found that the culture medium containing 10% fetal bovine serum has ~24.4 ng/ml of the 43-kd SPARC protein (Figure 4). As expected, high levels of SPARC secretion were seen for primary cell culture HOSE713 (5220 ng/ml) and immortalized HOSE1-15 cells (2600 ng/ml). Significantly reduced levels of secreted SPARC were detected for ovarian cancer cell lines SKOV3 (38.6 ng/ml), OVCA420 (15.3 ng/ml) and OVCA429 (149 ng/ml). Although still much lower than that of HOSE cells, DOV13 has the highest level of SPARC secretion (681.7 ng/ml). The amounts of SPARC secreted by the cell cultures directly correlate with their SPARC expression levels as detected by Western blot analysis of their total cell lysates (compare Figure 3 and 4).

Exogenous SPARC Inhibits the Proliferation of Ovarian Cancer Cells

To investigate the autocrine/paracrine effects of SPARC on ovarian cell growth, we have cultured HOSE1-15 and SKOV3 cells for 5 days in culture medium containing different concentrations of exogenous human platelet SPARC and monitored the amount of viable cells after incubation by MTT assay. This result is important, as it will indicate whether the platelet SPARC we used in this study produces the same growth inhibitory effect as the endogenous SPARC synthesized by HOSE cells. MTT assay showed that the number of viable HOSE 1-15 cells decreased to ~70% of the control when SPARC was added to the culture medium (Figure 5A). As for SKOV3 cells, the number of living cells decreased to ~66% of the control in the presence of 5 μg/ml of SPARC and reduced to ~54% of the control after being treated with 20 μg/ml of SPARC (Figure 5B). These results are consistent with the data obtained from BrdU incorporation ELISA. In this assay, SKOV3 and HOSE cells (5×10^3 cells/well) were incubated with different concentrations of SPARC in separate wells in a 96-well plate. After incubation at 37°C for 96 hours, the cells were labeled with the pyrimidine analogue BrdU for 6 hours. DNA synthesis was monitored based on the incorporation of BrdU into DNA, which is detected by immunoassay. Cellular proliferation of both HOSE cells and SKOV3 cells was reduced when treated with increasing amounts of SPARC (Figure 6), as indicated by the decrease in absorbance at 370 nm. The inhibitory effect of SPARC on SKOV3 cells is obviously greater than that on HOSE cells. Moreover, the overall BrdU incorporation is less in HOSE cells than in SKOV3 cells, probably because of the slower growth rate of the normal ovarian epithelial cells. Similar antiproliferative activity of SPARC was also observed for another two ovarian cancer cell lines OVCA433 and DOV13 (data not shown).

SPARC Induces Apoptosis in Ovarian Cancer Cells

Studies on transgenic and knockout mice have provided direct evidence that the disruption of apoptotic pathways in cells can lead to tumor development. To investigate the mechanism(s) that drives the antitumor activities of SPARC in ovarian cancer cells, we have examined whether SPARC can induce apoptosis in SKOV3 and HOSE cells. Induction of apoptosis after exogenous SPARC treatment was examined using the *in situ* cell death detection (TUNEL) assay and cell death detection ELISA (Roche Molecular Biochemicals).

In the TUNEL assay, SKOV3 cells (1×10^4 cells/well) were incubated with different amounts of SPARC at 37°C in 8-well chamber slides for 48 hours. After incubation, DNA strand breaks in the cells induced by apoptosis were detected by labeling the free 3′-OH DNA ends with fluorescein-labeled nucleotides using terminal deoxynucleotidyl transferase. For comparison, cell nuclei were also stained with DAPI regardless of the apoptotic status of the cells. Signals were visualized under fluorescence

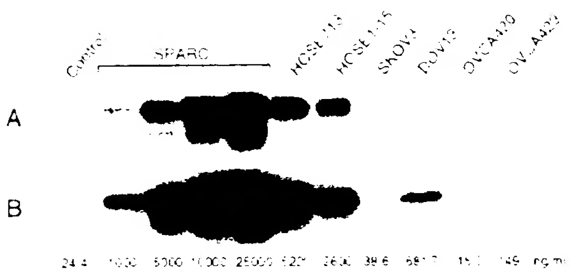

Figure 1. Western blot analysis of SPARC in the culture medium of normal HOSE cells and ovarian cancer cells. [illegible] **A:** [illegible] **B:** Longer exposure to show the signals detected [illegible] ovarian cancer cells.

microscope. Our results showed that untreated SKOV3 cells exhibit very low levels of spontaneous apoptosis frequently observed in cultured cells, which are barely visible in the figure (Figure 7A). When they were treated with 1 μg/ml of blood platelet SPARC, more apoptosis was detected (Figure 7C). Increased amounts of DNA strand breaks after treating SKOV3 cells with 5 μg/ml of SPARC were indicated by the increase in number and labeling intensity of the nuclei (Figure 7E). Cell nuclei stained with DAPI were detected as blue dots in all assays (Figure 7; B, D, and F). To clearly demonstrate the apoptotic effects of SPARC, we have selected areas on the slides in which more apoptotic cells were congregated. The estimated numbers of apoptotic SKOV3 cells detected after treating with SPARC were ~50 to 60% of the entire cell population examined. Our results showed that SPARC induces apoptosis in ovarian cancer cells.

To determine the apoptotic effects of SPARC on HOSE cells, cell death detection ELISA was used as a sensitive and quantitative assay for detecting DNA fragmentation. SKOV3 cells were also analyzed for comparison in this assay. SPARC-treated or untreated cells were used after incubation and the lysates were assayed for cytoplasmic histone-associated DNA fragments (mono- and oligonucleosomes). Increase in DNA fragmentation is indicated by the increase in absorbance at 405 nm. Consistent with the results obtained from TUNEL assays, SKOV3 cells showed increase in DNA fragmentation when treated with increasing amounts of SPARC (Figure 8B). When treated with 1 μg/ml of SPARC, a level much higher than that secreted by ovarian cancer cells (see Figure 4), SKOV3 cells showed a significant increase in DNA fragmentation when compared to the untreated control. In the presence of 5 μg/ml of SPARC, a concentration close to the normal range of SPARC secretion by primary HOSE cells (see Figure 4), DNA fragmentation is six times more than that of the control (Figure 8B). On the contrary, no significant increase in DNA fragmentation was seen when HOSE1-15 cells were treated with increasing amounts of SPARC (Figure 8A). No apoptotic effect was observed even after HOSE cells had been incubated with 20 μg/ml of SPARC (Figure 8A), which is much higher than their normal amounts of SPARC secretion (see Figure 4). Various extents of SPARC-induced apoptosis were also detected in four other ovarian cancer cell lines: DOV13, OVCA3, OVCA420, and OVCA429 (data not shown).

SPARC Binds to Normal and Cancerous Ovarian Cell Surface

The antiproliferative effects of SPARC on bovine aortic endothelial cells have been suggested to depend, in part, on signal transduction via a G protein-coupled re-

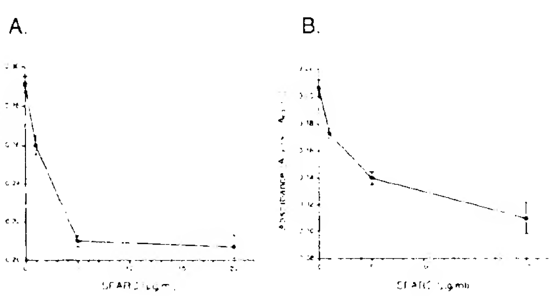

Figure 5. Exogenous SPARC inhibits the growth of HOSE1-15 and SKOV3 cells. [illegible]

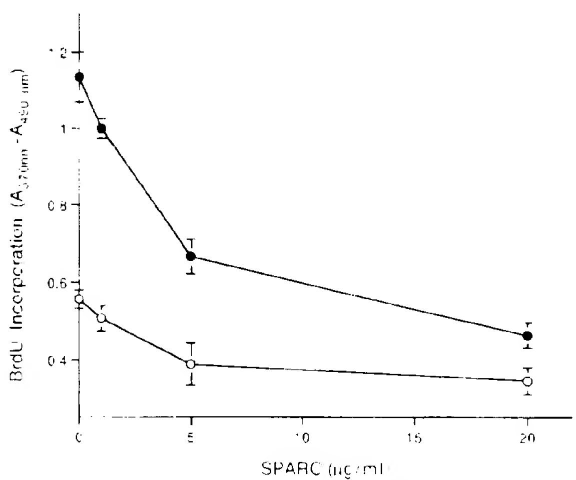

Figure 6. [illegible] **bars** [illegible] **Filled circles**, SKOV3; **open circles**, HOSE1-15.

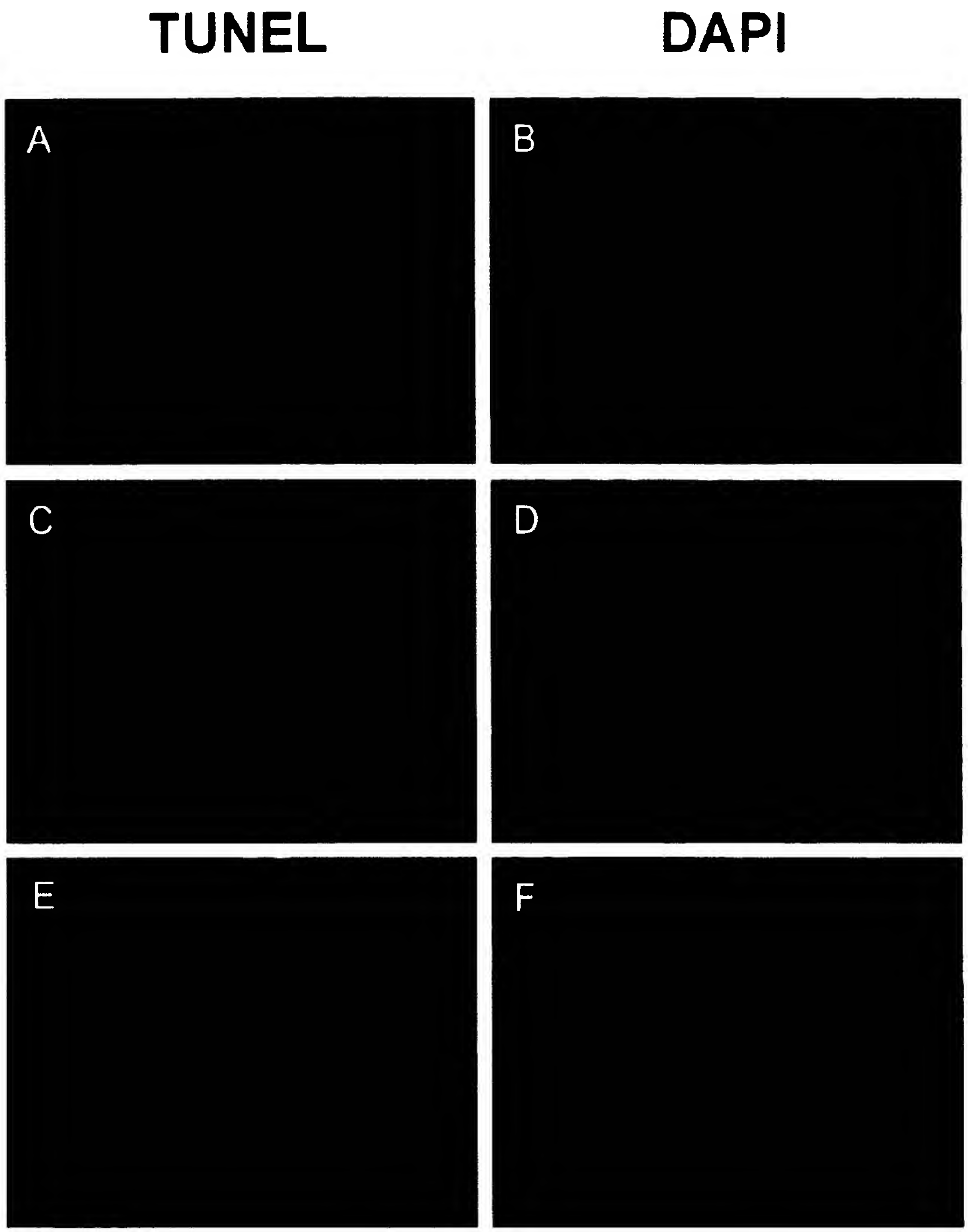

Figure 7. Detection of apoptotic ovarian cancer cells by TUNEL assay. SKOV3 cells seeded in chamber slides were treated with different concentrations of human blood platelet SPARC protein for 48 hours (**A** and **B**, untreated; **C** and **D**, 1 μg/ml; **E** and **F**, 5 μg/ml) [illegible]. TUNEL assays were performed (**A**, **C**, and **E**). To visualize the cell nuclei regardless of apoptosis, SKOV3 cells were also stained by DAPI (5 μg/ml) at room temperature for 10 minutes (**B**, **D**, and **F**). [illegible] apoptotic cells and DAPI-stained cells were detected by fluorescence microscopy. DAPI-stained cell nuclei were seen as [illegible] dots whereas the nuclei of apoptotic cells were stained green. There is a significant increase in the number and staining intensity of apoptotic cell nuclei when SKOV3 cells were treated with increasing amounts of SPARC, indicating more DNA strand breaks.

ceptor.[24] Together with the results we obtained from the treatment of HOSE and ovarian cancer cells with exogenous human blood platelet SPARC, we sought to find out whether the antiproliferative and apoptotic effects of SPARC on ovarian cells are also mediated through a cell surface receptor.

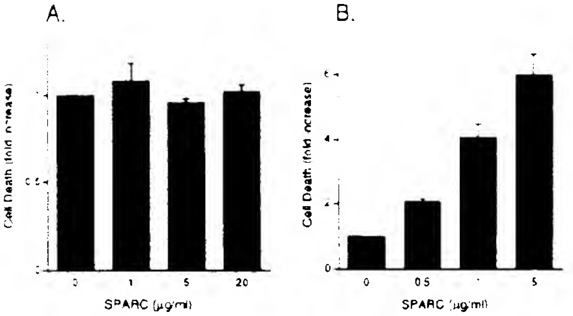

Figure 8. Detection of apoptosis in SPARC-treated HOSE cells and SKOV3 cells. A quantitative ELISA that detects mono- and oligo-nucleosomes was used to assay the whole cell lysates prepared from HOSE1-15 cells (**A**) and SKOV3 cells (**B**) treated with different amounts of exogenous SPARC. Increase in apoptosis is indicated by the increase in absorbance at 405 nm (reference wavelength 490 nm). SPARC effectively induces apoptosis in SKOV3 cells, but not in HOSE cells. The values shown are the means ± SEM (**bars**) of lupik re measurements in two independent experiments.

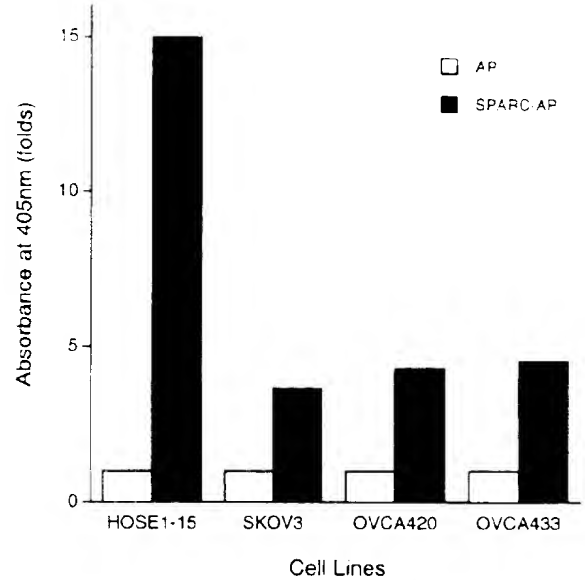

Figure 9. Cell surface receptor binding assays of HOSE cells and ovarian cancer cells. A secreted SPARC-AP fusion protein consists of human SPARC and a thermostable human placental alkaline phosphatase (AP) was used to examine the presence of putative SPARC receptors in ovarian cells. As background control, a secretory AP protein produced by the 293T pAPtag-4 stable cell line was also used in this assay. Cells incubated with culture medium containing SPARC-AP or AP proteins were washed, fixed, and assayed for AP activities. Increase in ligand binding is indicated by the increase in AP activity, which is quantified by measuring absorbance at 405 nm. HOSE1-15 cells showed high levels of binding, whereas the three ovarian cancer cell lines studied have less binding to the SPARC-AP fusion protein.

To examine whether putative SPARC receptors are present on ovarian cells, a secretory fusion protein containing full-length human SPARC and a thermostable human placental AP was made using a human embryonic kidney cell line (293T). As a negative control, culture medium containing high levels of secreted AP protein was also prepared from a 293T/pAPtag-4 stable cell line. Receptor binding assays using AP and SPARC-AP proteins revealed that putative SPARC receptors are present on both HOSE cells and ovarian cancer cells (Figure 9). The AP activities detected when the cells were incubated with the control AP protein are probably because of nonspecific binding of AP to cell surfaces and/or incomplete denaturation of the endogenous AP activities in the cells. Our results showed that in addition to the higher levels of SPARC expression, HOSE cells also have much more putative SPARC receptors on their cell surfaces (Figure 9).

Discussion

Using arbitrarily designed primers to generate differential RNA fingerprints from normal HOSE and ovarian carcinoma cells, we previously showed that SPARC is down-regulated in ovarian cancer.[25] Stable transfectants of ovarian cancer cells expressing high levels of SPARC grow slower and have greatly reduced ability to induce tumor formation in nude mice.[14] Nevertheless, the underlying mechanism(s) of these tumor-suppressing activities of SPARC is still unknown. Recent studies examining the immunoreactivity of SPARC in ovarian cancer tissues showed increased SPARC expression in ovarian cancer,[15,17] which are different from our earlier finding. To investigate this inconsistent pattern of SPARC deregulation in ovarian cancer, we studied a large number of ovarian tumor tissues of different grades and stages by immunohistochemistry using a rabbit polyclonal antibody to SPARC. This antibody, LF-54, has been tested to confirm its specificity and reactivity to bovine and human SPARC.[27] Our results revealed strong cytoplasmic immunoreactivity in the surface epithelial cells of human normal ovaries and benign epithelial tumors, which is progressively decreased in borderline epithelial tumors, and is significantly reduced or absent in invasive ovarian epithelial cancers. This strong direct correlation indicates that repression of SPARC expression is important in ovarian cancer development.

The strong immunoreactivity of SPARC in the germinal epithelium of normal ovary suggests that SPARC is important in maintaining the normal functions of ovarian surface epithelial cells. Its high expression in normal ovarian epithelial cells has also been detected by immunostaining using a monoclonal antibody, mAb SSP2, against a Ca^{2+}-binding region of murine SPARC.[16] Although SPARC was reported to be detected in 8 of 10 cases of ovarian carcinomas examined in that study,[16] no comparison of SPARC immunoreactivity of normal ovarian cells to that of benign, borderline, or invasive ovarian tumor was described. Our results presented here showed that although SPARC can still be detected in ovarian cancers, its expression is significantly down-regulated in high-grade ovarian cancers. The levels of expression are actually inversely correlated with the degree of malignancy.

In contrast to our findings, a recent study reported that SPARC can be detected in ovarian adenocarcinoma but not in ovarian surface epithelial cells by immunostaining using a monoclonal antibody generated against the N-terminal region of bovine SPARC (AON-5031).[17] Because SPARC expression could not be detected in the same

cancer tissues by *in situ* hybridization, the authors speculated that the SPARC found in cancer cells might be originated from stromal cells, as stromal cells in ovarian cancers have been shown to express SPARC (Porter et al,[18] Brown et al,[17] and this study). Although this hypothesis is interesting, it remains possible that the differences in SPARC immunoreactivity observed are because of the use of different antibodies and staining procedures. This is well illustrated by our immunostaining results using two different antibodies that showed distinctive patterns of SPARC immunoreactivity in ovarian tissues (compare Figures 1 and 2). Although the normal ovarian epithelium was strongly stained by LF-54, it was weakly stained with AON-5031. To the contrary, strong immunoreactivity was seen in the stroma of normal ovaries stained with AON-5031, but not in the sections stained with LF-54. In ovarian cancers, SPARC immunostaining was evident in the stromal cells, but rarely noted in the cancer cells with both the antibodies. This observation for ovarian tumor tissues agrees well with that previously reported by Paley and colleagues.[15] Based on these findings, the polyclonal antibody LF-54 might be more specific in human SPARC immunostaining. The immunohistochemical data we obtained with it are summarized in Table 1, which are consistent with our published results from Northern and Western blotting.[11]

The biological functions of SPARC seem to be variable in human cancers. Different tumors exhibit different patterns of SPARC expression. High levels of SPARC have been detected in several human cancers including melanoma,[28] breast cancer,[29] colorectal cancer,[30] hepatocellular carcinoma,[31] invasive meningioma,[32] and prostate cancer.[33] Moreover, it has been reported that SPARC promotes cell migration and invasion in prostate cancer and glioblastoma.[34,35] Suppression of SPARC expression by antisense RNA results in a significant decrease in the tumorigenicity of melanoma cells.[36] In contrast, no SPARC expression was found in chondrosarcoma, Ewing's sarcoma, fibrosarcoma, malignant fibrous histiocytoma, and brown tumor from hyperparathyroidism.[37] Decreased SPARC expression has also been found in ovarian cancer.[14] Additional evidence showing the tumor-suppressing activity of SPARC came from the studies of cultured cells. Significant down-regulation of SPARC was demonstrated in vSrc-transformed chicken embryo fibroblasts,[38] c-Jun-transformed primary rat embryo fibroblasts,[39] and Ha-Ras-, v-Abl-, v-Src-, or Ki-Ras-transformed rodent fibroblasts.[39-42] A recent study has also shown that SPARC strongly inhibits the growth of vJun-m1 and v-Src-transformed chicken embryo fibroblasts.[42] As different tumors are probably developed through different multistep carcinogenic pathways, these findings suggested that SPARC might play a tissue-specific role in cancer development.

The recent characterization of another SPARC-like counteradhesive extracellular matrix protein called Hevin/MAST9 further demonstrates the possible tumor-suppressing activities of matricellular proteins in human carcinogenesis. Hevin was found to be down-regulated in metastatic prostate adenocarcinoma and non-small cell lung cancer.[43,44] SPARC and Hevin are 62% identical in sequence and are highly homologous in the SPARC coding region.[44] Similar to SPARC, Hevin is also a secreted acidic cysteine-rich calcium-binding glycoprotein and has been shown to inhibit cell attachment and spreading.[45] The importance of these SPARC-like proteins in ovarian oncogenesis remains to be elucidated.

The antiproliferative activity of SPARC has been demonstrated in endothelial cells. Growth of normal endothelial cells is inhibited when cultured with medium conditioned by endothelioma cells that secrete high levels of SPARC.[22] Exogenous SPARC has also been shown to suppress DNA synthesis in bovine aortic endothelial cells and human microvascular endothelial cells stimulated by vascular endothelial growth factor.[46,47] The SPARC domain IV that contains an EF-hand-like loop and has a high-affinity Ca^{2+}-binding site is sufficient for the observed growth inhibitory functions.[23] The antiproliferative activity of SPARC was further illustrated by a recent study showing that the mesangial cells, fibroblasts, and smooth muscle cells isolated from SPARC-null mice grew faster than their respective wild-type counterparts.[18] Despite all these findings, no direct evidence is available showing the antiproliferative effects of SPARC on cancer cells. Here we show that exogenous SPARC can reduce the proliferation of both HOSE and ovarian cancer cells in a concentration-dependent manner. As HOSE cells secrete high levels of SPARC, the paracrine and/or autocrine antiproliferative activities of SPARC may play an important role in the precise regulation of normal HOSE cell growth. Diminished SPARC expression in ovarian cancer cells, together with other oncogenic factors, may lead to uncontrolled cell growth and cancer development.

Recent advances in basic cancer research have established that human cancers are the results of deregulation of not only the factors that control cellular proliferation and differentiation, but also those that influence apoptosis.[49] As a possible mechanism that contributes to the antitumor activities of SPARC, we presented here direct evidence that SPARC could induce apoptosis in ovarian cancer cells, but not HOSE cells. HOSE cells, which secrete high levels of SPARC, seem to have some mechanisms that protect themselves from the apoptotic activities of SPARC. This hypothesis is supported by a recent study showing that apoptosis can be inhibited in HOSE cells by the up-regulation of insulin-like growth factor-1, as mediated by luteinizing hormone/human chorionic gonadotropin signaling.[50] Follicle-stimulating hormone and human chorionic gonadotropin can both stimulate HOSE cell proliferation, but not SKOV3 cells.[51] As both follicle-stimulating hormone and luteinizing hormone receptors are highly expressed in HOSE cells, they were not detected in the gonadotropin insensitive SKOV3 cells.[51] A recent study has also reported that whereas HOSE cells show consistent expression of luteinizing hormone receptors, ovarian cancers exhibit a steady decrease in luteinizing hormone receptor expression from low-grade to high-grade cancer.[52]

As the growth rates of both HOSE cells and ovarian cancer cells were reduced by SPARC, our findings suggest that distinct signal transduction pathways are used

to mediate the antiproliferative and apoptotic effects of SPARC. Our preliminary results showed that SPARC could transiently trigger significant increases of intracellular Ca^{2+} levels in ovarian cancer cells, but not in HOSE cells, indicating that the apoptotic pathway induced by SPARC might be calcium-dependent. In addition, immunoblotting revealed that the expression of a pro-apoptotic protein was induced or significantly increased in SPARC-treated SKOV3 cells, but not HOSE cells (our unpublished data). We hypothesize that although down-regulation of SPARC in ovarian cancer cells may not be directly involved in oncogenesis, it is coupled to other oncogenic pathways that drive the development of ovarian cancer. Repression of SPARC expression may be essential to facilitate ovarian tumorigenesis, as cancer cells are sensitized to the apoptotic effects of SPARC. This concept is supported by the study showing that low levels of SPARC expression in v-Jun m1 and v-Src transformed chicken embryo fibroblasts favor their induction of local, primary fibrosarcomas after being subcutaneously injected into the wing web of chicken.[illegible]

Platinum derivatives such as cisplatin are routinely used in the chemotherapy of ovarian epithelial cancer. Studies of cisplatin-resistant ovarian cancer cells revealed that the observed chemoresistance might be caused by the expression of an apoptosis suppressor protein known as X-linked inhibitor of apoptosis protein (Xiap).[illegible] Cisplatin induces apoptosis in cisplatin-sensitive, but not cisplatin-resistant cells by decreasing Xiap expression.[illegible] Although it has yet to be determined, the apoptotic effect of SPARC on SKOV3 cells is unlikely mediated by the down-regulation of Xiap because SKOV3 cells have null p53 mutation. It has been shown that decreased Xiap expression could not induce apoptosis in SKOV3 cells because Xiap down-regulation triggers apoptosis only in ovarian cancer cells that have wild-type p53, but not in the cells with no or mutated p53 protein.[illegible]

The antiproliferative and apoptotic effects of exogenous SPARC on ovarian cancer cells indicate the presence of cell surface receptors for SPARC. This notion is supported by the results obtained from studying the mediators through which exogenous SPARC exerts its counteradhesive and antiproliferative effects on endothelial cells. Pretreating endothelial cells with protein tyrosine kinase inhibitors protected them against the inhibitory effect of SPARC on cell spreading. Moreover, inhibition of cell cycle progression by SPARC on these cells was found to be reversible by treating them with inhibitors for heterotrimeric G proteins, such as pertussis toxin and cholera toxin.[illegible] Our preliminary data also showed that cholera toxin (1 μg/ml) can reverse the growth inhibitory effect of SPARC by 41.5%, indicating the involvement of G-protein in SPARC signaling in ovarian cells (our unpublished data). To date, neither the putative SPARC receptor nor the intracellular signaling pathway(s) triggered by SPARC has been identified.

Using a fusion protein containing SPARC and human placental AP, we present here the first direct evidence that SPARC binds to putative SPARC receptors on the cell surfaces of HOSE and ovarian cancer cells. This AP-TAG technology has been used to successfully [illegible] the receptors for [illegible] and Semaphorin [illegible]. Our results showed that in addition to the down-regulation of SPARC expression, ovarian cancer cells have lower levels of SPARC receptor than HOSE cells. The binding of SPARC to its receptor may be important for mediating its antitumor effects. The diminished ligand-receptor interaction in ovarian cancer cells may explain why the growth of ovarian cancer is not influenced by the presence of low levels of SPARC produced by the adjacent stromal cells. Although HOSE cells have more putative SPARC receptors than ovarian cancer cells, their differential response to SPARC is probably caused by the distinctive downstream signaling events triggered by the binding of SPARC to its receptor as cancer cells have undergone numerous genetic changes during oncogenesis.

In this study, we showed that exogenous SPARC could reduce proliferation and induce apoptosis in ovarian cancer cells. Although distinct signaling pathways may mediate these tumor suppressing effects, they probably are dependent on the binding of SPARC to its cell surface receptor. Down-regulation of SPARC and/or SPARC receptor in ovarian cancer cells will decrease and interrupt normal SPARC ligand-receptor interaction, which in turn affects the downstream signaling events that are important for controlling the growth and differentiation of HOSE cells. Interaction of SPARC and its putative receptor, in addition to the various posttranslational modification of SPARC, may also contribute to the tissue- and cell-specific biological functions of SPARC in different normal and cancerous cells.

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The Effects of Particulate Wear Debris, Cytokines, and Growth Factors on the Functions of MG-63 Osteoblasts

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Background: Particle-challenged cells release cytokines, chemokines, and eicosanoids, which contribute to periprosthetic osteolysis. The particle-induced activation of macrophages and monocytes has been extensively studied, but only limited information is available on the response of osteoblasts to particulate wear debris. This study examines the effects of particulate wear debris, proinflammatory cytokines, and growth factors on osteoblast functions.

Methods: MG-63 osteoblasts were treated with metal particles (titanium, titanium alloy, and chromium orthophosphate) or polymeric particles (polyethylene and polystyrene) of phagocytosable sizes or were treated with exogenous cytokines and growth factors. The kinetics of particle phagocytosis and the number of engulfed particles were assessed with use of fluoresceinated particles. Cell proliferation was determined according to [^{3}H]-thymidine incorporation, and cell viability was determined by either fluorescein diacetate uptake or trypan blue exclusion. Expressions of osteoblast-specific genes were quantified with Northern blot hybridization, and the secretions of osteoblast-specific proteins and cytokines were analyzed by enzyme-linked immunosorbent assays.

Results: MG-63 osteoblasts phagocytosed particles and became saturated after twenty-four hours. A maximum of forty to sixty particles per cell were phagocytosed. Each type of particle significantly suppressed procollagen α1[I] gene expression ($p < 0.05$), whereas other osteoblast-specific genes (osteonectin, osteocalcin, and alkaline phosphatase) did not show significant changes. Particle-stimulated osteoblasts released interleukin-6 ($p < 0.05$) and a smaller amount of transforming growth factor-β1. Particles reduced cell proliferation in a dose-dependent manner without affecting cell viability ($p < 0.05$). Exogenous tumor necrosis factor-α also enhanced the release of interleukin-6 ($p < 0.01$) and transforming growth factor-β1 ($p < 0.05$), whereas the secretion of transforming growth factor-β1 was increased by insulin-like growth factor-I and prostaglandin E2 as well. Insulin-like growth factor-I and transforming growth factor-β1 significantly increased procollagen α1[I] gene expression in osteoblasts ($p < 0.05$), while tumor necrosis factor-α and prostaglandin E2 significantly suppressed procollagen α1[I] gene expression ($p < 0.01$). In contrast, neither exogenous nor endogenous interleukin-6 had any effect on other cytokine secretion, on proliferation, or on procollagen α1[I] gene expression. The transcription inhibitor actinomycin D reduced both procollagen α1[I] transcription and interleukin-6 production. Inhibitors of protein synthesis (cyclohexamide) and intracellular protein transport (brefeldin A and monensin) blocked the release of interleukin-6, but none of these compounds influenced the suppressive effect of titanium on procollagen α1[I] gene expression.

Conclusions: MG-63 osteoblasts phagocytose particulate wear debris, and this process induces interleukin-6 production and suppresses type-I collagen synthesis. Osteoblast-derived interleukin-6 may induce osteoclast differentiation and/or activation, but the resorbed bone cannot be replaced by new bone because of diminished osteoblast function (reduced type-I collagen synthesis). Exogenous cytokines (tumor necrosis factor-α and interleukin-1β), growth factors (insulin-like growth factor-I and transforming growth factor-β1), and prostaglandin E2 can modify particulate-induced alterations of osteoblast functions.

Clinical Relevance: Altered osteoblast functions probably contribute to the progression of periprosthetic osteolysis. Suppressed osteoblast functions, however, could be compensated for by certain growth factors, such as insulin-like growth factor-I or transforming growth factor-β1. These growth factors, if delivered locally, may have therapeutic potential to prevent or reverse periprosthetic osteolysis.

Periprosthetic osteolysis is a major clinical problem that may jeopardize the long-term success of total joint arthroplasty[1]. In periprosthetic osteolysis, a granulomatous tissue of fibroblasts, macrophages, and foreign-body giant cells develops at the interface of the bone and prosthesis or of the bone and bone cement[2-4]. All cell types of this interfacial tissue contain wear debris from prosthetic components[2-4], and particle phagocytosis is a central event in the pathogenesis of periprosthetic osteolysis[5-8].

Phagocytosis is a nonspecific defense mechanism for the elimination of tissue debris, bacteria, and foreign particles. The phagocytic process requires the opsonization of particles and a protein coat on the surface of particles that bind to phagocytosis receptors (such as Fcγ receptors, complement or mannose receptors, and β1 integrins). This interaction activates intracellular signaling pathways that lead to cytoskeletal reorganization, pseudopod formation, and the ingestion of the particles[19-21]. This phagocytosis-induced signaling process may simultaneously result in the upregulation or downregulation of a number of genes through the action of various nuclear transcription factors[9,22-24]. However, the normal phagocytic process may be altered when tissue macrophages or other cells are continuously exposed to nondegradable wear debris.

The phagocytosis of particulate wear debris stimulates macrophages/monocytes to secrete mediators of bone resorption such as eicosanoids, interleukin-1 (IL-1), tumor necrosis factor-alpha (TNF-α), and interleukin-6 (IL-6) *in vitro*[11,12,22,24,26,29]. These compounds have also been shown to be present in periprosthetic soft tissue *in vivo*[6,11,30].

Osteoblasts also phagocytose particles. This process upregulates the release of cytokines[25] and prostaglandin E2[31], inducing bone loss through osteoclast activation. In addition, particle-stimulated osteoblasts exhibit suppressed procollagen α1[I] gene expression followed by reduced type-I collagen synthesis[17,25], which may result in decreased formation of bone. Thus, particle-induced altered osteoblast functions may play a critical role in pathological bone resorption through both osteoclast activation and reduced osteoblastic bone formation.

In previous studies, we identified the upstream signaling events in particle-stimulated osteoblasts[25] that ultimately led to the suppression of procollagen α1[I] gene expression[17,25]. Activation of protein tyrosine kinases seems to be the earliest cellular event resulting in the activation of nuclear transcription factor-kappaB (NF-κB)[25] in osteoblasts, and this transcription factor was shown to be activated in particle-challenged human macrophages as well[24]. However, NF-κB is a general transcription factor that may suppress procollagen α1[I] mRNA[25,32] while simultaneously upregulating many other genes, including the genes of proinflammatory cytokines such as IL-1, IL-6, and TNF-α[24] in particle-challenged cells. Since these mediators are continuously secreted by particle-challenged cells, their effects may be crucial in the development of periprosthetic osteolysis by altering osteoblast and osteoclast functions.

We hypothesized that, besides the direct effects of particulate wear debris on osteoblasts (increased IL-6 release and suppressed type-I collagen synthesis)[25], there is a paracrine regulation of cytokines, prostaglandins, and growth factors, which contribute to bone resorption by alteration of osteoblast functions. We investigated this hypothesis by monitoring the kinetics of particle phagocytosis in osteoblasts and by determining the effects of proinflammatory cytokines and growth factors on osteoblast-specific gene expression, cell proliferation, and cytokine release.

Materials and Methods

Particles

All metal and polymeric particles used in this study were described previously[17,25,26]. Particles of commercially pure titanium with a 1 to 3-μm nominal diameter and chromium orthophosphate ($CrPO_4 \times 4H_2O$) (mean diameter and standard deviation, 1.42 ± 0.83 μm)[26] were purchased from Johnson Matthey (Danvers, Massachusetts). Titanium-alloy particles (6% aluminum and 4% vanadium) (Sulzer Metco, Troy, Michigan) were ground from 150 to 300-μm-sized grinding particles[25]. Conventional medical-grade ultra-high molecular weight polyethylene (GUR 415; Hoecht-Celanese, Houston, Texas) was pulverized in liquid nitrogen[25]. Particles were sedimented and then subjected to filtration. The particles had a comparable size distribution, with at least 90% of the particles less than 3 μm in diameter. Polystyrene particles (mean diameter and standard deviation, 1.14 ± 0.01 μm) and polystyrene-based fluorescent particles (Fluoresbrite) (mean diameter and standard deviation, 0.926 ± 0.027 μm) were purchased from Polysciences (Warrington, Pennsylvania). A 0.1% (volume/volume) particle suspension contained approximately 2.2 to 6.7 × 10^8 particles per milliliter. The particles were sterilized by irradiation with 2.2 megarad (22,000 gray) from a Cs-137 source (model 143; J.L. Shepherd Irradiator, San Fernando, California) opsonized in 10% human type-AB serum[26] and stored in sterile phosphate-buffered saline solution, pH 7.2. Endotoxin contamination of particles was excluded by limulus assay (E-Toxate; Sigma Chemical, St. Louis, Missouri).

Cells and Cell Cultures

The MG-63 osteoblast cell line was purchased from the American Type Culture Collection (ATCC, Rockville, Maryland). Cells were cultured in monolayer in Dulbecco modified Eagle medium (GIBCO, Grand Island, New York) containing 10% fetal bovine serum (HyClone Laboratories, Logan, Utah) in a humidified atmosphere of 5% carbon dioxide in air at 37°C[17,25,26].

Treatment of Cells with Particles, Cytokines, and Growth Factors

Confluent cultures of cells were subjected to serum starvation (0.3% fetal bovine serum) for twenty-four hours prior to treatment. Culture media were then replaced with fresh media consisting of 0.3% fetal bovine serum containing particles, cytokines, or growth factors. Proliferation and viability assays, phagocytosis analysis, and total RNA extraction were performed on the cultured cells. Tissue culture media were collected at various time-points, centrifuged, filtered through a 0.22-μm polycarbonate filter (Spin-x; CoStar, Cambridge, Massachusetts), and stored at −80°C. All of the experiments

were performed in duplicate or triplicate in at least five independent experiments.

Reagents were purchased from Calbiochem (La Jolla, California) or R and D Systems (Minneapolis, Minnesota). Insulin-like growth factor-I (IGF-I, 30 ng/ml) and transforming growth factor-beta1 (TGF-β1, 20 pg/ml) were used to stimulate collagen production[23,24]. Tumor necrosis factor-α, IL-6, and IL-1β were added to the cultures at concentrations of 10 ng/ml, 500 pg/ml, and 30 pg/ml. Prostaglandin E2 (100 ng/ml) is an eicosanoid that has been shown to regulate collagen synthesis[25]. Actinomycin D (1 μg/ml) was used to block transcriptional events, cyclohexamide (35.5 μM) was used to inhibit protein translation and synthesis, brefeldin A (0.1 μM) was used to inhibit the transport of freshly synthesized proteins from the endoplasmatic reticulum to the Golgi apparatus, monensin (1.5 μM) was used to block the release of newly synthesized proteins from the Golgi apparatus, and cytochalasin D (1 μM) was used to destabilize the cytoskeleton, thus inhibiting phagocytosis. All of the concentrations listed above were selected after serial dilutions of each compound were tested in MG-63 cell culture.

Viability Tests and [^{3}H]-Thymidine Incorporation

The trypan blue exclusion test was used to determine the viability of cells. Since the presence of phagocytosed titanium particles, especially at higher concentrations, precluded a precise evaluation of dye exclusion, cell viability was also determined with fluorescein diacetate (Molecular Probes, Eugene, Oregon)[26]. Viability tests were performed in duplicate, and at least 200 cells were counted with transmission or fluorescent microscopy in a Microphot-FXA microscope (Nikon, Tokyo, Japan).

Proliferation of cells was measured by the incorporation of [^{3}H]-thymidine (Amersham International, Arlington Heights, Illinois) into DNA in a ninety-six-well microplate system. Trypsinized cells were harvested (Cell Harvester; Tomtec, Orange, Connecticut) at different time-points after a twelve-hour [^{3}H]-thymidine (1 μC [37 Bcq] of [^{3}H]-thymidine per well) incubation.

RNA Extraction and Northern Blot Hybridization

Total RNA samples were isolated from monolayer cultures as described previously[27,28]. Approximately 10 μg of total RNA was denatured in 50% formamide and 17.5% formaldehyde, dissolved in MOPS buffer (20 mM 3-[N-morpholino]propanesulfonic acid, 5 mM sodium acetate, and 1 mM EDTA at pH 7.0), separated by electrophoresis in 1% agarose gel, and then transferred to GeneScreen Plus membranes (New England Nuclear, Boston, Massachusetts). Blots were hybridized with ^{32}P-deoxycytidine-triphosphate-labeled specific cDNA probes at a concentration of 3×10^6 cpm/ml (specific activities: 2 to 6×10^7 cpm/μg of cDNA)[18]. Human-specific cDNA probes (plasmids) were purchased from the American Type Culture Collection. The following recombinant plasmid DNAs were used as probes: a 1.8-kb cDNA probe for procollagen α1[I] (Hf677; ATCC 61322), a 2.0-kb probe for osteocalcin (ATCC 86269), a 1.8-kb probe for osteonectin (ATCC 78193), and a 1.5-kb probe for alkaline phosphatase (ATCC 59633). Following hybridization, the blots were washed and exposed to Kodak XAR-5 film (Eastman Kodak, Rochester, New York) at −70°C for photographic documentation or the original Northern blot was analyzed by STORM PhosphorImiger with ImigeQuant software (both from Molecular Dynamics, Sunnyvale, California).

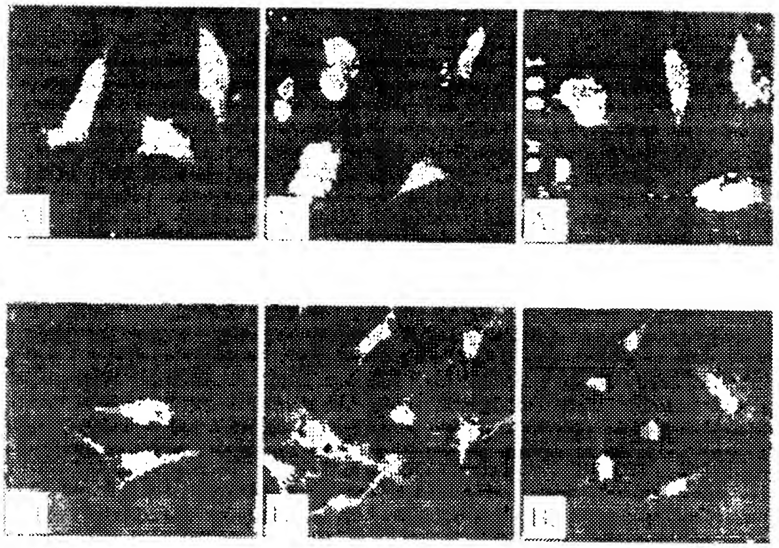
Measurement of Particle Phagocytosis

To further understand particle-induced changes in osteoblast function, we characterized the kinetics of particle uptake by MG-63 cells. In initial experiments, cells grown in monolayers were treated with 0.1% (volume/volume) titanium particles for various time-periods, harvested by trypsinization, washed, and allowed to attach to coverslips. Trypsinization and repeated washing of the cells removed nonphagocytosed and/or surface-attached particles, and cell-free areas of the coverslip had essentially no particles. Two hundred particle-treated cells from different areas on the coverslip were examined with light microscopy (Nikon). Although we could easily identify cells containing particles, it was difficult to determine the exact number of particles in a single cell because of the intracellular aggregation of titanium particles.

To circumvent this problem and to be able to quantify the number of phagocytosed particles within a single cell, we used fluorescent particles (Fluoresbrite) as an alternative source of particles. Two different methods were applied to determine the number of engulfed particles in osteoblasts. First, the number of Fluoresbrite particles in cells was counted directly in epifluorescence mode with use of a Microphot-FXA microscope. However, this method could only be applied to cells treated for short time-periods (less than twelve hours), as the large number of phagocytosed particles in cells treated for longer periods formed intracellular aggregates, precluding accurate particle counts. The second method involved preparing serial dilutions of Fluoresbrite particles. The particles were counted in a hemocytometer, and the fluorescence intensity of each concentration was determined with a fluorescent plate reader (Victor 1420 multilabel counter; Wallac, Gaithersburg, Maryland). The particle numbers and the corresponding fluorescence intensities were plotted to generate a standard curve, which then was used to determine the number of fluorescent particles (on the basis of the fluorescence intensities) in cell lysates from osteoblasts treated with Fluoresbrite. Cells containing phagocytosed Fluoresbrite were trypsinized, washed, counted, and lysed by ultrasonication (VirTis, Gardina, New York) at 20 kHz for two minutes on ice.

Measurement of Cytokines and Osteoblast-Specific Proteins in Culture Media

Cytokine concentrations in supernatants of osteoblast cultures were measured by sandwich enzyme-linked immunosorbent assays (ELISAs) in ninety-six-well plates. High-sensitivity assay kits for TNF-α (range, 0.5 to 32 pg/ml), IL-1β (range, 0.12 to 8.0 pg/ml), IL-6 (range, 0.12 to 8.0 pg/ml), and TGF-β1 (range, 31 to 2000 pg/ml) were purchased from R and D Systems. Secreted osteocalcin was measured by NovoCalcin and type-I collagen was measured by Procollagen-C ELISAs purchased from Metra Biosystems (Mountain View, California).



Statistical Analysis

Descriptive statistics were used to determine group means and standard deviations. The Pillai trace (similar to Wilks lambda or the Hotelling-Lawley trace) criterion was used to detect multivariate significance. Subsequently, paired Student t tests were performed between groups of interest. The level of significance was set at $p < 0.05$. All statistical analyses were performed with use of computer-based statistical software (SPSS/PC+, version 4.0.1; SPSS, Chicago, Illinois).

Results

Particle Phagocytosis by Osteoblasts

Osteoblasts phagocytosed particles in a time-dependent fashion (Fig. 1-C). Determining the number of phagocytosed particles, however, is difficult because of the indiscernible location of phagocytosed, partially engulfed, or surface attached particles and the intracellular aggregation of phagocytosed particles. The use of fluorescent particles (Fluoresbrite) is a method for determining the number of engulfed particles. The mean number of particles per cell (and standard deviation), measured with fluorescence intensity in MG-63 cell lysates, was 0.4 ± 0.6 (range, zero to two) after one hour, 5 ± 2 (range, three to twelve) after two hours, 13 ± 3 (range, seven to nineteen) after six hours, 23 ± 2 (range, sixteen to twenty-eight) after twelve hours, 53 ± 5 after twenty-four hours, 60 ± 6 after forty-eight hours, and 58 ± 5 after seventy-two hours (Fig. 1-C). A maximum of forty to sixty Fluoresbrite particles phagocytosed within twenty-four hours seems to be the saturation level for MG-63 cells. The number of engulfed particles could be precisely determined only when fluorescent-labeled particles were

used. Cytochalasin D, which destabilizes the cytoskeleton and inhibits phagocytosis, significantly reduced ($p < 0.01$) the phagocytosis of particles (4 ± 3 particles per cell after forty-eight hours) but did not completely abolish it (Figs. 1-A, panel A2, and 1-B, panel B2).

Effect of Particulate Wear Debris on Cell Viability and Proliferation

Next, we addressed how the phagocytosed particles influenced cell functions and whether different compositions of particles could initiate different cell responses. Particulate wear debris had no effect on the viability of MG-63 cells, which remained higher than 95% even in long-term experiments (seventy-two to ninety-six hours) over a wide range of particle compositions (titanium, titanium alloy, chromium orthophosphate, polyethylene, polystyrene, and Fluoresbrite) and concentrations (0.0125% to 0.2% volume/volume). In contrast to viability, cell proliferation was reduced when compared with that in untreated cultures, and the effect was dose-dependent (Fig. 2, A). Interestingly, suppressed proliferation returned to normal by forty-eight hours at low particle concentrations (0.0125% to 0.05%) (Fig. 2, A).

Effect of Exogenous Cytokines on Cell Viability and Proliferation

Exogenous cytokines (IL-1β and IL-6), prostaglandin E2, and growth factors TGF-β1 and IGF-I had no effect on cell viability over a wide range of concentrations. Only TNF-α significantly reduced cell viability when higher concentrations (more than 100 ng/ml) were used ($p < 0.01$), especially in long-term experiments (more than seventy-two hours). As shown in Figure 2, *B*, TNF-α (at a nontoxic concentration of 10 ng/ml) and prostaglandin E2 decreased cell proliferation, while TGF-β1 and IGF-I increased it. Neither IL-β1 nor IL-6 affected cell proliferation.

Cytokine Release in Osteoblasts Induced by Either Titanium Particles or Exogenous Cytokines

The earliest cytokine release in culture media of titanium-

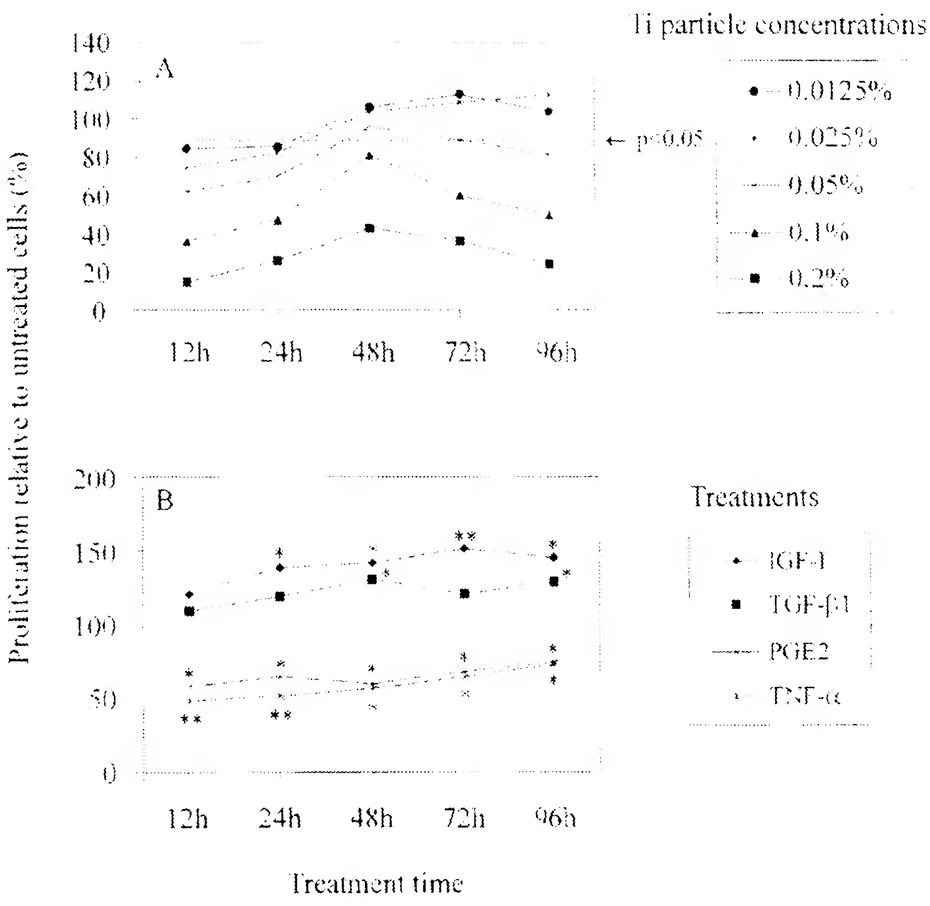


Fig. 2

Effect of titanium particles and exogenous mediators on MG-63 cell proliferation. Cells were not treated or were treated either with different concentrations of titanium particles (panel A) or with exogenous cytokines and growth factors (panel B). The level of proliferation was measured with $[^3H]$ thymidine incorporation and was normalized to untreated samples (100%) at each time point. Error bars are omitted for clarity, but the broken line indicates significance at a minimum level of $p < 0.05$ in panel A. In panel B, * indicates a level of significance (relative to untreated cells) of $p < 0.05$ and ** indicates a level of $p < 0.01$.

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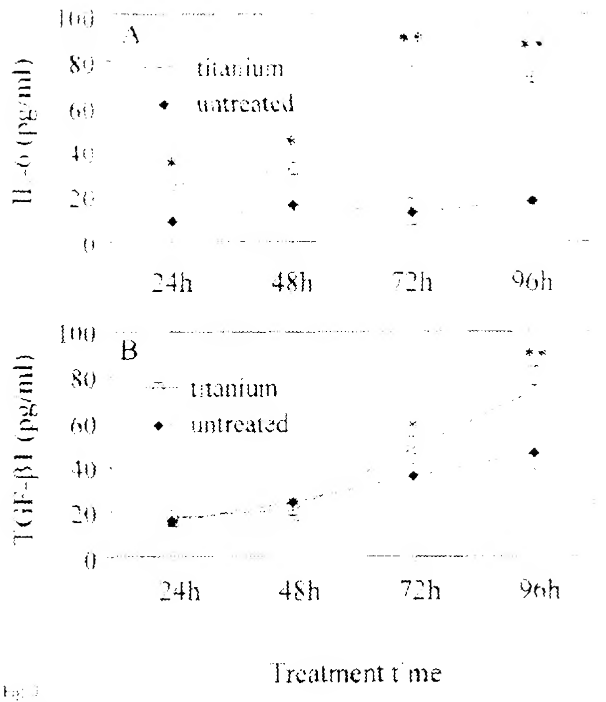

Fig. 3

Effect of titanium particles on production of IL-6 and TGF-β1 by MG-63 cells. Confluent osteoblast cultures were not treated or were treated with 0.1% (volume/volume) titanium particles. Levels of IL-6, TNF-α, IL-1β, and TGF-β1 were measured with enzyme-linked immunosorbent assays at various time-points. Only IL-6 (panel A) and TGF-β1 (panel B) reached detectable and significantly elevated levels, with * indicating a p value of less than 0.05 and ** indicating a p value of less than 0.01.

stimulated MG-63 cells was detected after twelve hours, and it was restricted to IL 6 (Fig. 3, *A*). There were undetectable amounts of IL-1 and TNF-α in culture media of either untreated or particle-challenged MG-63 cells. A basal TGF-β1 secretion was enhanced in particle-treated cultures after seventy-two hours (Fig. 3, *B*).

As found in titanium-stimulated MG-63 cultures, only IL-6 and TGF-β1 secretion was modified by exogenous mediators. Only TNF-α at a nontoxic concentration (10 ng/ml) had a significant effect on IL-6 release ($p < 0.01$) (Fig. 4, *A*), whereas the basal TGF-β1 secretion was increased ($p < 0.05$) by exogenous TNF-α, IGF-I, or prostaglandin E2 (Fig. 4, *B*).

Suppression of Osteoblast-Specific Gene Expression

We reported a 40% to 60% suppression of procollagen α1[I] mRNA expression in MG 63 osteoblasts exposed to titanium particles[1-3]. To further characterize the effect of particles on osteoblast-specific gene expression and protein synthesis, MG-63 cells were exposed to titanium, titanium-alloy, chromium orthophosphate, polystyrene, polyethylene, and Fluoresbrite particles for forty-eight hours. Particles, regardless of composition, significantly suppressed procollagen α1[I] mRNA expression in MG-63 osteoblasts ($p < 0.05$) (Fig. 5). This down-regulation of collagen gene expression was accompanied by reduced type I collagen protein synthesis. In contrast to procollagen gene expression, none of the particles significantly altered the expression of osteocalcin or other osteoblast-specific genes such as osteonectin or alkaline phosphatase. These data demonstrate that particles differentially affect gene expression in osteoblasts (for example, procollagen α1[I] compared with osteocalcin) (Fig. 5), and the gene-specific effect is a general response to particles and is not specific to particles of a particular composition.

Effect of Exogenous Cytokines on Procollagen α1[I] Gene Expression

An increased IL-6 secretion in titanium-stimulated osteoblasts (Fig. 3, *A*) correlated inversely with the suppression of procollagen α1[I] mRNA and reduced collagen synthesis in all particulate-stimulated osteoblast cultures. Neither exogenous IL-6 (Fig. 6, *A*) nor neutralizing antibodies to IL-6 (data not shown) altered the collagen gene expression in the presence or absence of titanium particles, indicating that procollagen α1[I]

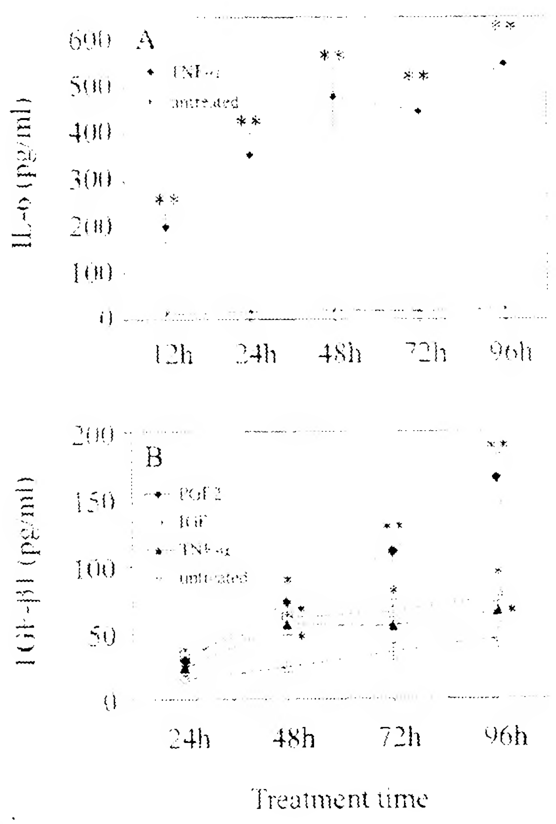

Fig. 4

Effect of exogenous cytokines on the release of cytokines by MG-63 cells. Confluent osteoblast cultures were not treated or were treated with exogenous IL-6, TNF-α, IL-1β, TGF-β1, IGF-I, or prostaglandin E2 as described in Materials and Methods. Conditioned media were collected at various time-points, and cytokine levels were measured with enzyme-linked immunosorbent assays. Only IL-6 (panel A) and TGF-β1 (panel B) reached detectable and significantly elevated levels, with * indicating a p value of less than 0.05 and ** indicating a p value of less than 0.01.

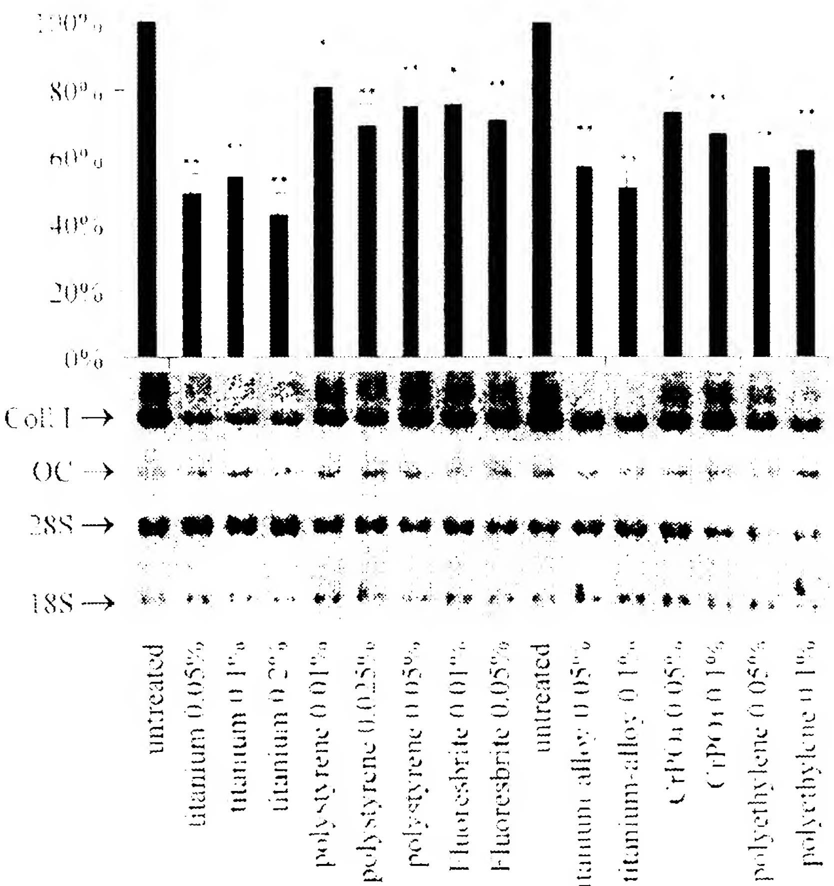

Fig. [illegible]

The effect of various particles on procollagen α1[I] and osteocalcin gene expressions in MG-63 cells. Confluent cell cultures were deprived of serum for twenty-four hours and were left untreated (lanes 1 and 10) or were treated with different particles for forty-eight hours at the various concentrations indicated. The level of gene expression was compared with that in untreated samples. Columns represent means of duplicates of at least five independent experiments (and the standard deviation) of procollagen α1[I] gene expression. * indicates a p value of less than 0.05, and ** indicates a p value of less than 0.01. The osteocalcin gene expression in particle-treated MG-63 cells showed no significant differences, and a representative hybridization panel (OC) is shown. All types of particles significantly reduced the procollagen α1[I] mRNA expression.

gene regulation was independent of IL-6 or IL-6-induced transcription factors in osteoblasts. In contrast, the effect of exogenous TNF-α on procollagen α1[I] gene expression was highly comparable with the effect of titanium particles (Fig. 6, *A*), and this correlated with reduced type-I collagen synthesis[5]. Prostaglandin E2 also inhibited procollagen α1[I] gene expression (data not shown). Exogenous IL-1β significantly reversed titanium-induced procollagen α1[I] gene suppression ($p < 0.05$) (Fig. 6, *A*). Growth factors IGF-I and TGF-β1 significantly increased collagen gene expression ($p < 0.05$) and could completely reverse the titanium-induced suppression of procollagen α1[I] mRNA (Fig. 6, *B*).

Effect of Transcriptional, Translational, and Protein Transport Inhibitors on Cytokine Release and Procollagen α1[I] Gene Expression

To determine whether an effect of a freshly synthesized cytokine was involved in the titanium-induced procollagen α1[I] gene suppression and to distinguish the mechanism of titanium-induced IL-6 production from procollagen α1[I] gene suppression at the molecular level, MG-63 cells were treated with various inhibitors prior to stimulation with titanium particles. Actinomycin D, a potent inhibitor of transcriptional events by inhibiting RNA polymerase II, was used as a positive control. This compound blocked both procollagen α1[I] mRNA transcription and IL-6 production. Translational (protein-synthesis) inhibitor cyclohexamide, protein-transport inhibitor brefeldin A, and monensin (a nonselective inhibitor of the release of newly synthesized protein from the Golgi apparatus) uniformly blocked the release of IL-6. In contrast, none of these chemicals modified titanium-particle-induced suppression of the procollagen α1[I] mRNA level (Fig. 7), confirming our recent observation that particle phagocytosis has a direct effect on procollagen gene expression through the activation of the protein tyrosine kinase-NF-κB pathway[7]. Taken together, particle phagocytosis has a direct effect on procollagen α1[I]

mRNA, whereas particle induced cytokine release requires factors for protein synthesis and intracellular trafficking.

Discussion

Particulate wear debris from prosthetic components is continuously generated and phagocytosed by cells of the periprosthetic soft tissue. Phagocytosis is a strong signal for cells, first inducing a series of upstream events of cell stimulation through the activation of protein tyrosine kinases [illegible]. The activation of protein tyrosine kinases leads to the activation of nuclear transcription factors. These nuclear transcription factors then are translocated into the nucleus, resulting in the upregulation of various genes, including proinflammatory cytokines. In a broader sense, all cell types of the periprosthetic soft tissue (macrophages, fibroblasts, osteoclasts, and osteoblasts) are able to phagocytose particulate wear debris, and virtually all cells can reach an activated state. These cells produce a number of cytokines, chemokines, and prostaglandins, which may further affect the function of cells in either an autocrine or a paracrine manner with use of distinct signaling mechanisms.

Bone is a dynamic tissue with a well-balanced homeostasis preserved by both formation and resorption of bone. Normal turnover of bone, however, can be unbalanced by either increased osteoclast activity or decreased osteoblast function; either mechanism or both mechanisms may result in a net loss of bone. Both osteoclasts and osteoblasts phagocytose particles *in vitro*, and it is assumed that this process may occur *in vivo* as well. Osteoblasts, which phagocytose particles, become activated and produce IL-6 [illegible] and prostaglandin E2 [illegible], simultaneously losing their capacity to synthesize type-I collagen [illegible]. The secreted IL-6 [illegible] and prostaglandin E2 [illegible] then activate osteoclasts in a paracrine fashion, which are assumed to already be in an activated state because of phagocytosed particles at the interface [illegible]. Other cytokines such as IL-1β and TNF-α, secreted by particle-stimulated macrophages/monocytes [illegible], are also present in the periprosthetic tissue. Tumor necrosis factor-α can activate osteoblasts to secrete IL-6 and suppress type-I collagen synthesis [illegible], an effect similar to that described for particle phagocytosis (Figs. 3, A; 4, A; and 6, A). In addition, both IL-1β and TNF-α induce osteoclast differentiation from precursors and activate differentiated osteoclasts *in vitro* [illegible]. Taken together, phagocytosis directly affects and phagocytosis-induced cytokine release indirectly affects the bone turnover negatively by altering osteoblast and osteoclast functions.

From the osteoblast side, a phagocytosis-induced direct signal and exogenous TNF-α (paracrine effect) seem to be the most potent inducers of diminished type-I collagen synthesis. However, the particle induced and TNF-α-induced signaling mechanisms must be independent because (1) we were unable to detect TNF-α in either particle treated MG 63 (Fig. 3) or

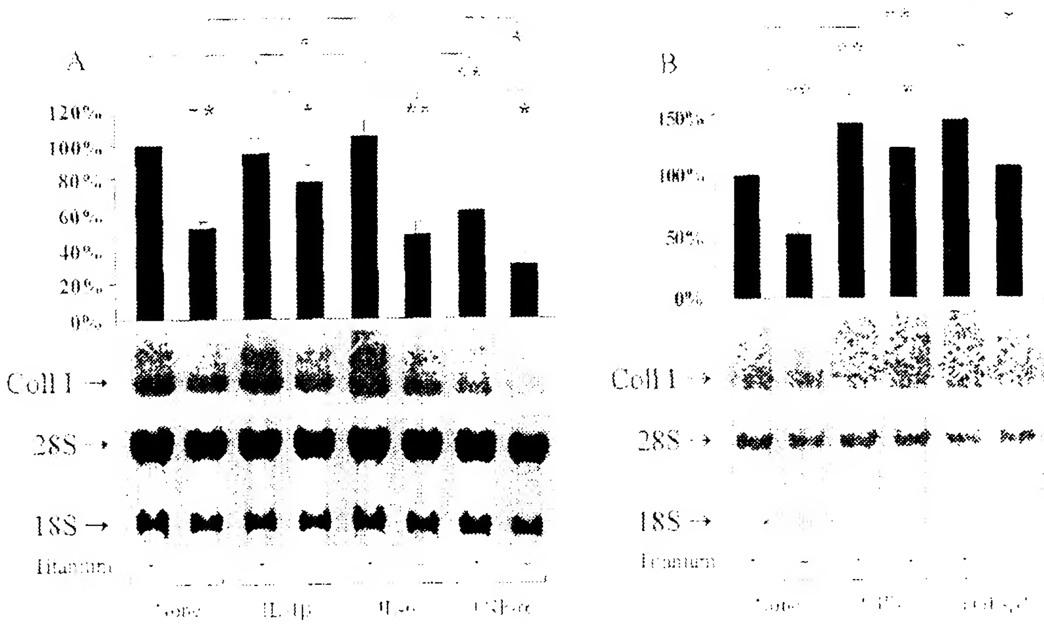

Fig. 6

Northern blot analysis of procollagen α1(I) mRNA expression in MG 63 cells treated with different cytokines (panel A) or growth factors (panel B) in the presence or absence of phagocytosable-size titanium particles. Confluent osteoblast cultures were deprived of serum for twenty-four hours and were pretreated with cytokine/growth factor for three hours, and then titanium particles (0.1% volume/volume) were added where indicated. Bottom panels show the amount of total (ribosomal) RNA on an ethidium bromide-stained membrane prior to hybridization. [illegible] represent means of duplicates of at least five independent experiments and the standard deviation. Data are normalized to the mRNA level measured in nonstimulated MG 63 cells (lane 1 in both panels). * indicates a p value of less than 0.05, and ** indicates a p value of less than 0.01. Neither IL-6 nor IL-1β had an effect on procollagen α1(I) gene expression in the particle-free condition, although IL-1β could partially compensate for the suppressive effect of titanium on procollagen α1(I) gene expression.

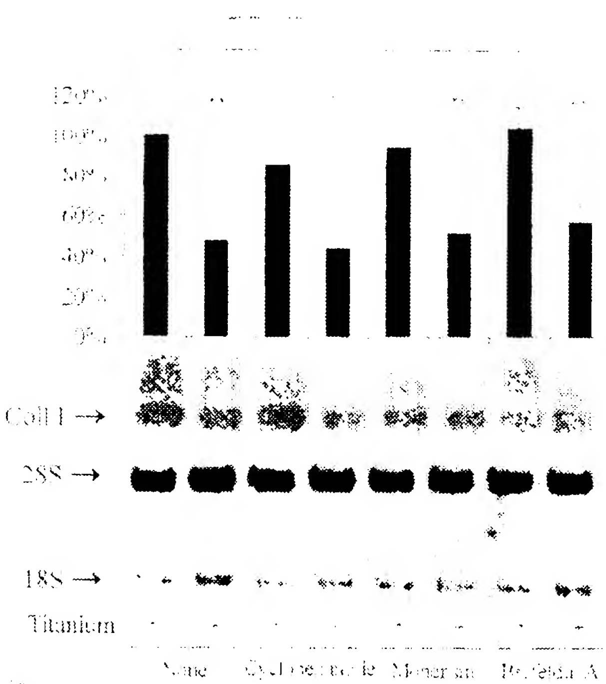

Fig. 7

Effect of protein synthesis and transport inhibitors on procollagen α1(I) gene expression. Confluent MG-63 osteoblast cultures were deprived of serum for twenty-four hours and were left untreated or were pretreated with the indicated compound for three hours, and then titanium particles (0.15% volume/volume) were added where shown. The level of gene expression was analyzed by Northern blots to determine procollagen α1(I) mRNA and was compared with that of untreated samples (lane 1). Columns represent means of duplicates of at least five independent experiments and the standard deviation. * indicates a p value of less than 0.05, and ** indicates a p value of less than 0.01. None of the compounds were able to reverse the particle-treatment-induced gene suppression.

bone-marrow-derived primary human osteoblast cultures[25], (2) neutralizing anti-TNF-α antibody could abolish the exogenous TNF-α effect but did not modify the osteoblast response to titanium particles (data not shown), and (3) protein synthesis inhibitors, while blocking cytokine release, had no influence on particle-induced procollagen α1(I) gene suppression (Fig. 7).

While neither exogenous nor endogenous IL-6 can affect osteoblast-specific functions, IL-6 along with the IL-6 soluble receptor could enhance different osteoblast responses[47-49]. The IL-6 receptor complex consists of two transmembrane proteins: a ligand-binding chain (IL-6 receptor) and a non-ligand-binding signal transducer, glycoprotein 130 (gp130). Interleukin-6 binding to the ligand-binding chain triggers heterodimerization of the two chains, and then the cytoplasmic domain of the gp130 chain transduces the signal. The soluble form of the ligand-binding chain (soluble IL-6 receptor) is also able to activate gp130 when IL-6 binds to it. It is likely that MG-63 osteoblasts express gp130 but not the ligand-binding chain[47,50]. Therefore, neither secreted nor exogenous IL-6 can bind to the IL-6 receptor. As a result, no signal can be transferred from the cell surface to the cell[47,50].

Among a number of cytokines and growth factors, IGF-I and TGF-β1 were able to completely reverse the suppressive effect of particles on procollagen α1(I) gene expression. These growth factors, when used alone, significantly upregulated the procollagen α1(I) gene expression (Fig. 6, B) and type I collagen synthesis. Furthermore, these growth factors increased osteoblast proliferation without affecting cell viability or inducing substantial IL-6 secretion. Thus, IGF-I and TGF-β1 seem to be potent inducers of bone matrix formation.

One of the most important findings of the present study is that, in addition to a direct effect of particles on osteoblast functions[5-7], the proinflammatory cytokine TNF-α also exhibits a massive and substantial effect on procollagen α1(I) gene expression, cell proliferation, cell viability, and IL-6 secretion in osteoblasts. While this proinflammatory cytokine induces bone resorption via osteoclast activation, it also contributes to bone loss via reduced bone formation by osteoblasts. Since TNF-α, IL-1β, and IL-6 are present and are continuously secreted by particle-stimulated cells in the periprosthetic space, their long-term *in vivo* autocrine and paracrine effects are critical in the pathogenesis of the periprosthetic osteolysis. Eventually, local delivery of certain growth factors (IGF-I or TGF-β1), protein tyrosine kinase, or NF-κB inhibitors[25]—all of which can reverse the suppressive effect of either proinflammatory cytokines or wear particles on type-I collagen synthesis in osteoblasts—may have the therapeutic potential to prevent or treat periprosthetic bone loss. ■

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High pressure effects on cellular expression profile and mRNA stability. A cDNA array analysis

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Abstract. Hydrostatic pressure has a profound effect on cartilage tissue and chondrocyte metabolism. Depending on the type and magnitude of pressure various responses can occur in the cells. The mechanisms of mechanotransduction at cellular level and the events leading to specific changes in gene expression are still poorly understood. We have previously shown that induction of stress response in immortalized chondrocytes exposed to high static hydrostatic pressure increases the stability of heat shock protein 70 mRNA. In this study, our aim was to examine the effect of high pressure on gene expression profile and to study whether stabilization of mRNA molecules is a general phenomenon under this condition. For this purpose a cDNA array analysis was used to compare mRNA expression profile in pressurized vs. non-pressurized human chondrosarcoma cells (HCS 2/8). mRNA stability was analyzed using actinomycin-treated and nontreated samples collected after pressure treatment. A number of immediate-early genes, and genes regulating cell cycle and growth were up-regulated due to high pressure. Decrease in osteonectin, fibronectin, and collagen types VI and XVI mRNAs was observed. Also bikunin, cdc37 homologue and Tiam1, genes linked with hyaluronan metabolism, were down-regulated. In general, stability of down-regulated mRNA species appeared to increase. However, no increase in mRNA above control level due to stabilization was noticed in the genes available in the array. On the other hand, mRNAs of certain immediate-early genes, like c-jun, jun-B and c-myc, became destabilized under pressure treatment. Increased accumulation of mRNA on account of stabilization under high pressure conditions seems to be a tightly regulated, specific phenomenon.

1. Introduction

Articular cartilage functions as load-bearing tissue and has to withstand high compressive loads. The extracellular matrix produced by chondrocytes is resilient and mediates forces between moving bones. Rising from a chair produces a nearly 20 MPa pressure peak and during walking pressures cycle between atmospheric and 3–4 MPa at a frequency of 1 Hz [7]. During other activities the pressure can be even higher. Chondrocytes respond to mechanical forces by altering their production of extracellular components and by remodeling the cartilage tissue. Thus immobilization leads to loss of cartilage proteoglycans within a few weeks, and remobilization can for the most part reverse this event [12]. On the other hand, excessive load can permanently damage cartilage and alter the cellular activity which has been shown in various models of joint instability *in vivo* [4].

*R.K.S. and H.M.K. contributed equally to this work.

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Moderate level of hydrostatic pressure increases the synthesis of cartilage matrix molecules [6,11,14], while high continuous pressure results in inhibition of synthesis [6,10]. Under high hydrostatic pressure, heat shock protein 70 accumulates in the pressurized cells [8,16]. This accumulation involves stabilization of the corresponding mRNA [8], an event that appears to require protein neosynthesis [9]. Expression profiling of chondrocytic cells under pressure revealed a number of other genes whose expression was affected by high hydrostatic pressure [13].

For the moment, studies of mechanical forces on chondrocyte metabolism have mainly focused on measuring the changes in the synthetic activity of chondrocytes in tissue or cell cultures in response to various loading protocols. Many hypotheses concerning the possible mechanotransduction and mechanoreceptors have been suggested, however, analyses of how mechanical force affects gene expression have received less attention. In this study we have analyzed the alterations of gene expression in a human chondrosarcoma cell line on account of high continuous hydrostatic pressure and its possible effects on mRNA stability using a cDNA array.

2. Materials and methods

2.1. Cell culture and pressure treatment

HCS2/8 human chondrosarcoma cell line [17] was cultured in a humidified 5% CO_2/95% air atmosphere at 37°C in DMEM (Gibco, Paisley, UK) supplemented with 10% fetal calf serum (PAA, Linz, Austria), penicillin (50 units/ml, PAA), streptomycin (50 units/ml, PAA), and 3 mM glutamine (PAA). Cells were grown to subconfluent state before the experiments. Before exposure to hydrostatic pressure, medium was changed and 15 mM Hepes (pH 7.3) was added. To expose the cells to pressure, the culture dishes were filled with the medium described above and sealed with a plastic membrane. The apparatus for hydrostatic pressurization of the cells has been previously described in detail [11]. The pressure level of the test chamber was selected to be 30 MPa and static mode of pressure loading was used. Samples from control and pressurized cultures were collected after pressurization for 6 hours. To study the mRNA stability, actinomycin D (2.5 mg/ml, Sigma, St. Louis, MO, USA) was dissolved in methanol and added to control and pressurized cultures at final concentration of 10 μM for 5 hours, and the samples were collected for mRNA isolation.

2.2. Expression profiling with a cDNA array

The Atlas Human Cancer 1.2 cDNA array kit was purchased from Clontech Laboratories (Palo Alto, CA, USA). All procedures for labeling and purification of the probes were accomplished according to manufacturer's recommendations. α-^{32}P-dATP-labeled cDNA probes were generated by reverse transcription of mRNA from untreated and pressurized monolayer cultures using gene-specific primers. Unincorporated label was removed by column chromatography. The membranes were hybridized in ExpressHyb^{TM} solution overnight at 68°C, washed twice in 0.2 × SSC and 0.1% sodium dodecyl sulfate (SDS), and twice in 0.1 × SSC, 0.5% SDS, for 20 min each. Autoradiography signals were quantified by using a PhosphorImager^{TM} (Molecular Dynamics, Sunnyvale, CA, USA), and the values obtained were normalized against several housekeeping genes.

3. Results

Continuous 30 MPa hydrostatic pressure was applied on chondrosarcoma cells to compare the mRNA expression profile in the pressurized cells with control cultures using the cDNA array technique. Since our goal was to investigate the mRNA stability as well, we pressurized the cells for 6 hours, a time interval needed to reach maximal heat shock protein 70 mRNA level and stabilization [8]. A number of changes in mRNA expression levels was observed due to high pressure. The up-regulated genes are presented in Table 1, and down-regulated ones in Table 2. The house-keeping genes (GAPDH, α-tubulin, β-actin, 40S ribosomal protein, 23-kDa highly basic protein and HLAC) were expressed consistently, and their average was used for the normalization.

To study changes in mRNA stability, possibly affected by high hydrostatic pressure, mRNA synthesis was stopped by addition of actinomycin D in control and pressurized cultures, and expression profiles were compared with corresponding RNA samples which were collected immediately after pressurization. A number of up-regulated (Table 1) and down-regulated (Table 2) genes were observed. Changes in signal levels between actinomycin D-treated and untreated array samples were considered to indicate that mRNA half-life differed from the average half-life of total mRNA pool (Tables 1 and 2). Ratio 1.0 represents the case where the mRNA half-life did not change.

4. Discussion

The immediate early genes, such as c-jun, c-myc, jun-B, some G-proteins and transcription factors were observed to be induced effectively during pressure treatment. Ets transcription factors are involved in cartilage and bone development, and type X collagen, e.g., has a conserved Ets-binding sequence in its promoter region [2]. Therefore, increase in Elf mRNA may have relevance in chondrocyte's response to pressure. mRNAs for c-jun, c-myc and jun-B were present at rather low level in control cell cultures, however, they had a long half-life, as indicated by increase of their relative abundance in actinomycin D treated cultures. Interestingly, even though pressurization resulted in a notable increase of these mRNA species, their half-life appeared to be shortened in pressurized cells. This seems to indicate faster turnover rate of the early phase messages. The signal of these genes seemed to be intense even 6 hours after the beginning of pressurization indicating ongoing activation of other target genes.

Under high hydrostatic pressure, a number of genes involved in cell cycle regulation were up-regulated. Effects of pressure on the cell cycle have been previously studied with *Saccharomyces cerevisiae*, where hydrostatic pressure in the range of 15 to 25 MPa caused arrest of the cell cycle in G_1 phase, while pressure of 50 MPa did not [1]. Although the expression profiling suggests that 30 MPa hydrostatic pressure would result in cell cycle arrest in the chondrosarcoma cells, as indicated by increased signal of p21 and Gadd45, no direct evidence is available for an involvement of apoptotic process. In fact, many of the mRNA species involved in apoptosis were decreased in the pressurized cells.

Histone H4 signal was slightly increased in pressurized chondrosarcoma cells under study, while in HeLa cells all histone mRNA levels dropped at 41 MPa hydrostatic pressure applied for 10 min [15]. However, the differences in cell type and duration of the loading may explain these contradicting results. Destabilization of histone mRNA was noticed in both experiments. Continuous 30 MPa pressure decreases aggrecan production in primary chondrocytes [10], and similar effect on mRNAs of matrix molecules osteonectin, fibronectin and collagen type VI was seen here. Hyaluronan has an important role

Table 1

Genes detected on cDNA array which were up-regulated and/or whose mRNA stability was changed due to high hydrostatic pressure

Gene	Fold of change a	Fold of change b	Fold of change c	Function	GB access number
c-jun	41.9	20.4	0.2	proto-oncogene	J04111
E4BP4 gene	13.7	5.2	0.7	transcription factor	X64318
growth arrest and DNA damage protein 45 (Gadd 45)	9.7	1.9	0.9	DNA repair, stress response	S40706
heparin-binding EGF-like growth factor (HBEGF)	8.9	3.6	1.4	growth factor	M60278
c-myc oncogene	8.1	8.8	0.2	proto-oncogene	V00568
nerve growth factor-inducible PC4 homologue	7.2	2.9	0.3	growth factor, cytokine	Y10313
jun-B	5.9	4.3	0.3	proto-oncogene	M29039
early growth response gene alpha (EGR-α)	5.9	4.6	0.7	transcription regulator	S81439
CDC-like kinase (CLK-1)	5.0	3.0	0.6	cell cycle-regulating kinase	L29222
NGF-inducible anti-proliferative protein PC3	4.4	6.4	0.6	cell cycle-regulating kinase	U72649
Rho8 protein	4.0	4.1	1.1	G-protein, signal transduction	X95282
EGF-response factor 1 (ERF-1)	3.6	3.3	0.8	transcription regulator	X79067
early growth response protein 1 (hEGR1, KROX 24)	3.3	2.8	0.6	transcription regulator	X52541
Gem (Ras-like protein KIR)	3.1	3.9	0.8	G-protein, signal transduction	U10550
DNA-binding protein CPBP	2.8	1.4	0.4	DNA binding protein	U44975
RBQ1 retinoblastoma binding protein	2.7	1.1	0.5	cell cycle, transcription	X85133
tyrosine-protein kinase ABL2 (tyrosine kinase ARG)	2.7	1.8	2.2	proto-oncogene	M35296
B4-2 protein	2.7	1.9	0.7	morphogenesis	U03105
MCL-1	2.5	3.1	1.7	bcl family protein	L08246
vascular endothelial growth factor (VEGF)	2.5	2.4	0.9	growth factor	M32977
CDK-interacting protein (cip1, waf1, p21)	2.4	1.4	1.1	cell cycle-regulating protein	U09579
platelet-derived growth factor A (PDGF-1)	2.2	1.4	0.9	growth factor	X06374
fos-related antigen (fra-1)	2.1	2.7	1.0	proto-oncogene	X16707
cyclin-dependent kinases regulatory subunit 2 (CKS-2)	2.1	1.3	1.0	cell cycle-regulating kinase	X54942
fos-related antigen 2 (fra-2)	2.1	3.2	1.0	proto-oncogene	X16706
ets-related transcription factor Elf-1	2.0	1.4	1.5	transcription activator	M82882
activator 1 37-kDa subunit	1.6	2.4	0.5	replication factor	M87339
STAT induced STAT inhibitor-3	1.5	4.7	0.6	kinase inhibitor	AB004904
wee-1 like protein kinase	1.5	2.6	0.4	cell cycle-regulating protein	U10564
histone H4	1.3	1.7	0.5	histone	X67081
interleukin-17 receptor	1.2	2.1	0.9	interferon receptor	U58917
bone morphogenetic protein 4 (BMP-4)	1.2	6.2	1.6	growth factor	D30751
polyhomeotic 2 homolog (HPH2)	1.2	2.2	1.1	transcriptional regulator	U89278

a) Fold of increase in expression under 30 MPa hydrostatic pressure for 6 h.
b) Relative mRNA stability in control sample (actinomycin-treated control vs. control).
c) Relative mRNA stability in pressurized sample (actinomycin-treated pressurized sample vs. pressurized sample).

in cartilage matrix linking tens to hundreds of aggrecan molecules into proteoglycan aggregates. mRNAs coding for three hyaluronan-associated proteins were down-regulated by high pressure, too. Placental bikunin is essential for biosynthesis of inter-α-trypsin inhibitor heavy chain-hyaluronan complex [18], and cdc37 has been shown to bind to hyaluronan [5]. Tiam1 and hyaluronan receptor CD44 interaction stimulates Rac1 signaling and hyaluronan-mediated breast tumor cell migration [3]. Decrease in mRNAs coding both H and M chains of lactate dehydrogenase suggest changes in carbohydrate metabolism in pressurized cells.

Table 2

Genes detected on cDNA array which were down-regulated and/or whose mRNA stability was changed due to high hydrostatic pressure

Gene	Fold of change			Function	GB access number
	a	b	c		
protein kinase C inhibitor	0.6	0.6	1.4	kinase inhibitor	U51004
tissue inhibitor of matrix metalloproteinase 2 (TIMP-2)	0.6	0.8	1.7	protease inhibitor	J05593
c-myc binding protein	0.6	0.7	1.4	inhibitor of myc-activity	D89667
osteonectin (SPARC, BM-40)	0.6	0.9	2.0	extracellular matrix protein	J03040
cyclin-dependent kinases regulatory subunit 1 (CKS-1)	0.5	0.8	1.7	cell cycle-regulating kinase	X54941
guanylate kinase (GMP kinase)	0.5	0.8	1.6	cGMP cycling	L76200
active breakpoint cluster region-related protein	0.5	0.6	1.3	GTPase-activating protein	U01147
hepatoma-derived growth factor (HDGF)	0.5	0.9	2.0	growth factor	D16431
signal transducer and activator of transcription 3 (STAT3)	0.5	1.1	2.0	transcription factor	L29277
L-lactate dehydrogenase H chain	0.5	0.6	1.5	carbohydrate metabolism	Y00711
fibronectin	0.5	0.8	2.0	extracellular matrix protein	X02761
TAX1-binding protein TXB151	0.5	0.7	2.1	transcriptional activator	U33821
procollagen α_1(VI)	0.5	0.8	2.8	extracellular matrix protein	X15879
von Hippel–Lindau tumor suppressor protein	0.5	1.2	1.8	tumor suppressor	L15409
extracellular signal-regulated kinase 6 (ERK-6)	0.5	0.8	0.9	signal transduction	X79483
GTP-binding protein ras-associated with diabetes (RAD1)	0.5	0.7	1.5	G-protein, signal transduction	L24564
procollagen α_1(XVI)	0.5	0.7	1.8	extracellular matrix protein	M92642
fatty acid synthase	0.5	1.0	1.8	lipid metabolism	S80437
acid finger protein	0.5	1.9	2.3	DNA binding	U09825
CHD3	0.5	0.8	1.9	transcription factor	AF006515
γ-tubulin	0.5	0.9	1.5	cytoskeletal protein	M61764
L-lactate dehydrogenase M chain	0.5	1.0	1.7	carbohydrate metabolism	X02152
placental bikunin, inter-α-trypsin inhibitor light chain	0.5	0.8	1.9	protease inhibitor	U78095
replication protein A 70 kDa subunit	0.5	0.7	1.7	replication factor	M63488
nucleoside 5'-diphosphate phosphotransferase	0.5	0.8	1.5	nucleotide metabolism	Y07604
PRSM1 metalloproteinase	0.4	1.0	1.5	metalloproteinase	U58048
growth-arrest specific protein (GAS)	0.4	0.7	1.9	growth suppression	L13720
procollagen α_3(VI)	0.4	1.0	2.0	extracellular matrix protein	X52022
insulin-like growth factor binding protein 6 (IGFBP6)	0.4	0.7	1.5	modulation of IGF activity	M62402
CDC37 homolog	0.4	0.8	1.5	cell cycle regulation	U63131
BCL7B protein	0.4	1.3	2.0	cytoskeletal protein	X89985
smoothened	0.4	0.8	1.6	morphogenesis	U84401
dishevelled 1 (segment polarity protein)	0.4	0.8	1.6	morphogenesis	U46461
IMP synthetase (inosinicase)	0.4	1.2	1.4	purine metabolism	U37436
inosine phosphorylase (PNP)	0.4	0.9	2.3	nucleotide metabolism	X00737
extracellular signal-regulated kinase 2 (ERK-2)	0.4	0.8	1.4	signal transduction	L11285
TRAP1	0.4	0.8	1.6	death-receptor	U12595
transforming growth factor-α (TGF-α)	0.4	1.0	1.7	growth factor	K03222
chromatin assembly factor 1 p48 subunit	0.4	0.8	1.6	chromatin protein	X74262
branched-chain amino acid aminotransferase	0.4	0.8	1.8	amino acid metabolism	U68418
T-lymphoma invasion and metastasis inducing (Tiam1)	0.4	0.7	2.0	oncogene	U16296
death-associated protein-1 (DAP-1)	0.3	0.7	2.4	death-receptor	X76105
Wnt-5a	0.3	1.0	2.1	morphogenesis	L20861

Table 2
(Continued)

Gene	Fold of change			Function	GB access
	a	b	c		number
PCNA	0.3	1.1	1.6	cell cycle-regulation	M15796
Fas-activated serine/threonine (FAST) kinase	0.3	0.8	2.1	death kinase	X86779
vaccinia-related kinase 2	0.3	1.1	1.8	intracellular kinase network	AB000450
integrin-linked kinase (ILK)	0.2	0.9	2.1	signal transduction	U40282
apoptosis regulator bax	0.2	0.5	4.5	apoptosis	L22474

a) Fold of decrease in expression under 30 MPa hydrostatic pressure for 6 h.
b) Relative mRNA stability in control sample (actinomycin-treated control vs. control).
c) Relative mRNA stability in pressurized sample (actinomycin-treated pressurized sample vs. pressurized sample).

Expression of heat shock protein 70 is an example of accumulation of mRNA solely due to mRNA stabilization without increased transcription in cells grown under high pressure [8]. In this experiment that kind of stabilization pattern was not observed with other genes, which indicates the uniqueness of the heat shock response under pressure. The overall picture given by this array analysis thus indicates that high static hydrostatic pressure influence on the amount of expression products involved in cell cycle regulation, transcription factors regulating various other genes and also various matrix molecules. The stability of up-regulated genes as a whole is somewhat decreased and involves no clear stabilization. In contrast, the stability of down-regulated genes seems to be somewhat increased.

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Identification of Proteins Secreted by Human Osteoblastic Cells in Culture

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ABSTRACT

To better understand the biochemistry of matrix-forming cells, we developed a simple and reproducible procedure for the isolation and identification by N-terminal sequencing of proteins secreted by cells into culture medium and applied this procedure to the analysis of the major Coomassie blue-staining proteins under 100 kD that are secreted from three different human osteoblastic cell cultures. The major proteins secreted by normal human osteoblasts from adult trabecular bone were identified by N-terminal sequencing to be gelatinase, osteonectin, the C-terminal propeptides of the α_1 and α_2 chains of type I collagen, tissue inhibitor of metalloproteinase 1 (TIMP-1), and β_2-microglobulin. The amounts of each of these proteins secreted into medium over a 24 h interval did not change over the 7 consecutive days of culture under serum-free conditions, which indicates that this pattern of protein secretion is not significantly affected by the serum-free conditions needed for protein identification by this method. In addition, radioimmunoassay for bone gla protein (BGP), a marker for osteoblast phenotype, revealed that BGP secretion remained high over 7 days of culture under serum-free conditions and was comparable to the rate of BGP secretion in control cultures with 10% serum. The major proteins secreted by MG-63 cells were identified by N-terminal sequencing to be gelatinase, a novel 40 kD human bone protein we termed YKL-40, TIMP-1, the recently discovered TIMP-2, and β_2-microglobulin. Further studies revealed that YKL-40 is the only protein detectable by Coomassie staining of SDS gels of MG-63 media proteins that is induced by extended time at confluence or by treatment with 1,25-$(OH)_2D_3$. The apparent absence of detectable Coomassie-stained bands corresponding to the C-terminal propeptides of collagen in the medium of MG-63 cells suggests that these transformed cells may not be a good model for bone matrix formation. The major proteins secreted by normal fetal osteoblastic cells were identified by N-terminal sequencing to be osteonectin and the C-terminal propeptides of the α_1 and α_2 chains of type I collagen. Gelatinase and TIMP could not be detected among the conditioned medium proteins by these methods. These observations indicate that fetal osteoblasts primarily express proteins that are matrix constituents and adult human osteoblasts secrete, in addition to these, proteins that could function in matrix turnover.

INTRODUCTION

A COMPLETE UNDERSTANDING of the physiologic function of a given cell requires knowledge of the identity and amount of each protein secreted by that cell. To date, two methods have been employed to identify proteins secreted by bone cells and by other extracellular matrix-forming cells. Proteins have been isolated from the extracellular matrices of tissues, and the expression of given proteins by matrix-forming cells has been assessed by immunologic assays for antigen and by cDNA hybridization assays for mRNA. These procedures have several limitations. For example, the isolation of proteins from the extracellular matrix is limited to the identification of secreted

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proteins that become abundant constituents of that matrix, and the identification of individual proteins expressed by matrix-forming cells is limited to the specific assays available for use in detection. Neither of these methods therefore identifies all proteins secreted by a matrix-forming cell. In the present study, we focused on the development of general methods for the separation and identification of proteins secreted by cells in culture and applied these methods to the biochemical characterization of human osteoblastic cells.

Three different bone cell cultures were used in this study. One is the MG-63 human osteosarcoma cell line, a cell that displays such osteoblastic characteristics as the secretion of the bone-specific protein bone gla protein (BGP; osteocalcin) in response to 1,25-dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$] stimulation.[1] The other two cell cultures were normal osteoblasts that we isolated from fetal and adult human bone for use in this study. Although fetal human bone cells have not been extensively studied, osteoblastic cells from adult human bone have been shown to secrete BGP in response to 1,25-$(OH)_2D_3$ treatment[2] and to synthesize gelatinase,[3] tissue inhibitor of metalloproteinase (TIMP),[3] bone sialoprotein,[4] osteonectin,[5] the small bone proteoglycans biglycan and decorin,[6] thrombospondin,[7] and type I collagen.[5,8] In each of these previous studies, the identification of proteins synthesized by adult human osteoblastic cells in culture was based on either immunologic detection of antigen or cDNA hybridization assays for mRNA. In the present study we identified by N-terminal protein sequencing the major Coomassie blue-staining proteins under 100 kD that are secreted by each of these three bone cell cultures. The identification of these proteins provides further insight into the possible biochemical functions of osteoblasts.

MATERIALS AND METHODS

Materials

The MG-63 human osteosarcoma cell line was obtained from the American Type Culture Collection (Rockville, MD). Coon's F12 (Ham's formulation) medium, RPMI medium, Dulbecco's modified Eagle's medium (DMEM), newborn calf serum, antibiotics, and trypsin were obtained from Irvine Scientific. 1,25-Dihydroxyvitamin D_3 was a gift from Dr. M.R. Uskokovic at Hoffman-LaRoche. Trifluoroacetic acid for high-performance liquid chromatography (HPLC) was from Pierce, HPLC-grade acetonitrile was from Fisher, sequencer reagents were obtained from Applied Biosystems, and electrophoresis reagents were from BioRad. All other reagents were analytic reagent grade or better.

Cell culture

Adult human bone cells were established in culture by a modification of the procedure of Gehron Robey and Termine[9] from the trabecular bone of each of 10 different femoral heads removed from women aged 60–86 years during surgery for hip replacement. None had any metabolic or malignant bone disease documented at the time of surgery. In a typical experiment, trabecular bone was cut into small pieces (10–20 mm^3) and washed thoroughly with DMEM and Ham's F12 medium (50:50 vol/vol) containing no serum to remove nonadherent marrow cells. The bone pieces were then incubated in the same serum-free medium containing 1 mg/ml of crude collagenase (type IV, Sigma Chemical Company) at 37°C with rotation. After 2 h the bone pieces were washed three times with DMEM and Ham's F12 containing 10% newborn calf serum to inhibit collagenase, and 8–12 of the collagenase-treated bone pieces were placed in each 100 mm plate and cultured in the same medium. Cells began to migrate from the bone pieces onto the plate after 2 weeks and typically reached confluence at 1 month. At this time, bone pieces were removed and the cells were trypsinized and divided into four 100 mm plates (passage 1). All subsequent subcultures were carried out by dividing confluent 100 mm plates into two 100 mm plates. Adult bone cells were routinely grown in DMEM and Ham's F12 medium (50:50 vol/vol) containing 10% newborn calf serum, 100 U/ml of penicillin, 100 μg/ml of streptomycin, 1 μg/ml of vitamin K_1, and 50 μg/ml of vitamin C. Cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2. To obtain conditioned medium for analysis, adult bone cells (passages 1 and 2) were grown to confluence in 100 mm dishes, the culture medium was removed, and the cell layer was washed twice with 10 ml phosphate-buffered saline. Serum-free DMEM and Ham's F12 (10 ml) containing 10 ng/ml of 1,25-$(OH)_2D_3$, 50 μg/ml of vitamin C, and 1 μg/ml of vitamin K_1 was then added to each dish. Conditioned medium was decanted 1 day later from each dish and replaced with 10 ml fresh serum-free medium containing the same level of added constituents. This procedure was repeated daily for up to 7 days. Conditioned medium was freed of cells and debris by centrifugation and frozen. Since the first 24 h conditioned medium sample sometimes contained traces of the bovine serum protein fetuin, all N-terminal sequences reported here were determined on proteins isolated from conditioned medium collected starting with day 2.

Fetal human bone cells were established from the calvaria of a 20-week-old human fetus (miscarriage) by the same modified procedure of Gehron Robey and Termine[9] as used for adult cells. Fetal bone cells were routinely cultured in Coon's F12 medium containing 10% newborn calf serum, 100 U/ml of penicillin, 100 μg/ml of streptomycin, 1 μg/ml of vitamin K_1, and 50 μg/ml of vitamin C. Cultures were incubated at 37°C in a humidified atmosphere of 10% CO_2. Although the present investigations were carried out using fetal bone cells that had undergone 10 population doublings from primary culture, these cells maintain their morphology and growth rate for at least 20 population doublings. To obtain conditioned medium for analysis, fetal bone cells were grown to confluence in 100 mm dishes, the culture medium was removed, and the cell layer was washed twice with 10 ml phosphate-buffered saline. Serum-free Coon's F12 media (10 ml) containing 10 ng/ml of 1,25-$(OH)_2D_3$, 50 μg/ml of vitamin C, and 1 μg/ml of vitamin K_1 was then added to each dish. Conditioned medium was collected daily in the same manner as described for adult bone cells.

The MG-63 human osteosarcoma cell line was cultured in 100 mm dishes with RPMI-1640 medium containing 10% newborn calf serum, 100 U/ml of penicillin, 100 μg ml of streptomycin, 50 μg/ml of vitamin C, and 1 μg/ml of vitamin K_1. Cultures were incubated at 37°C in a humidified atmosphere of 10% CO_2. At confluence, the culture medium was removed and the cell layer was washed twice with 10 ml phosphate-buffered saline. Serum-free RPMI-1640 (10 ml) containing 10 ng/ml of 1,25-$(OH)_2D_3$, 50 μg ml of vitamin C, and 1 μg/ml of vitamin K_1 was then added to each dish. Conditioned medium was collected daily in the same manner as described for adult bone cells

Preparative C4 reversed-phase HPLC

In the experiment described in Fig. 6, 3.5 liters conditioned media collected over 7 days of culture in the absence of serum was filtered through a 0.45 μm cellulose nitrate filter and loaded directly onto a Vydac C4 preparative reversed-phase column (2.2 cm inner diameter × 25 cm) at a flow rate of 10 ml/minute. After the sample was loaded, the column was washed for 10 minutes with 0.1% trifluoroacetic acid (TFA) in H_2O at a flow rate of 5 ml/minute. Next a linear gradient was developed from 0.1% TFA in H_2O to 0.1% TFA in 60% acetonitrile (vol/vol) over 2 h at a flow rate of 5 ml/minute. Fractions (1 minute) were collected and 200 μl aliquots of every other fraction were dried in a Speed Vac concentrator (Savant) for sodium dodecyl sulfate (SDS)-gel electrophoresis.

SDS-polyacrylamide gel electrophoresis

Electrophoresis was performed as described by Laemmli[9] using a Mini Gel Apparatus (BioRad Laboratories, Richmond, CA) and 0.75 mm thick gels. BioRad low-molecular-weight standards were run to calculate the apparent molecular weights of protein bands. Fractions from preparative C4 reversed-phase chromatography were concentrated for electrophoresis by freeze-drying in a Speed Vac concentrator. Medium from all other experiments was dialyzed exhaustively against Milli-Q water (Millipore Corp., Bedford, MA) at 4°C for 48 h using tubing with a 3500 molecular weight cutoff (Spectrapor 3; Spectrum Medical Industries, Inc., Los Angeles, CA) and freeze-dried in a Speed Vac concentrator. Dried proteins were dissolved in SDS sample buffer[9] containing 5% by

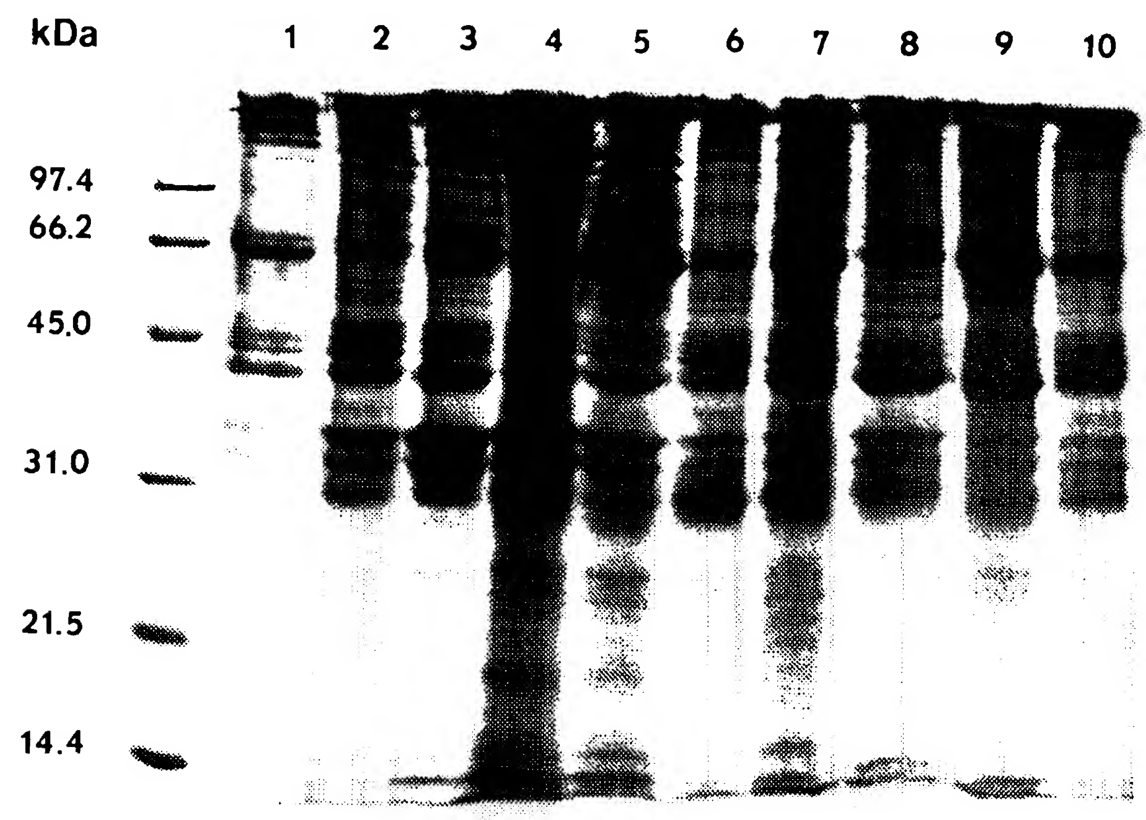

FIG. 1. SDS-polyacrylamide gel analysis of proteins secreted from adult human bone cells from 1 · different bone donors. Cells were grown to confluence in the presence of 10% newborn calf serum and then changed · · serum-free medium. Conditioned medium (6 ml) from the second day of serum-free culture was dialyzed and dried · · each of the 10 donors. The dried medium was dissolved in 20 μl SDS loading buffer, loaded onto a lane of a 12.5 · SDS polyacrylamide gel, electrophoresed, and stained with Coomassie blue (see Materials and Methods).

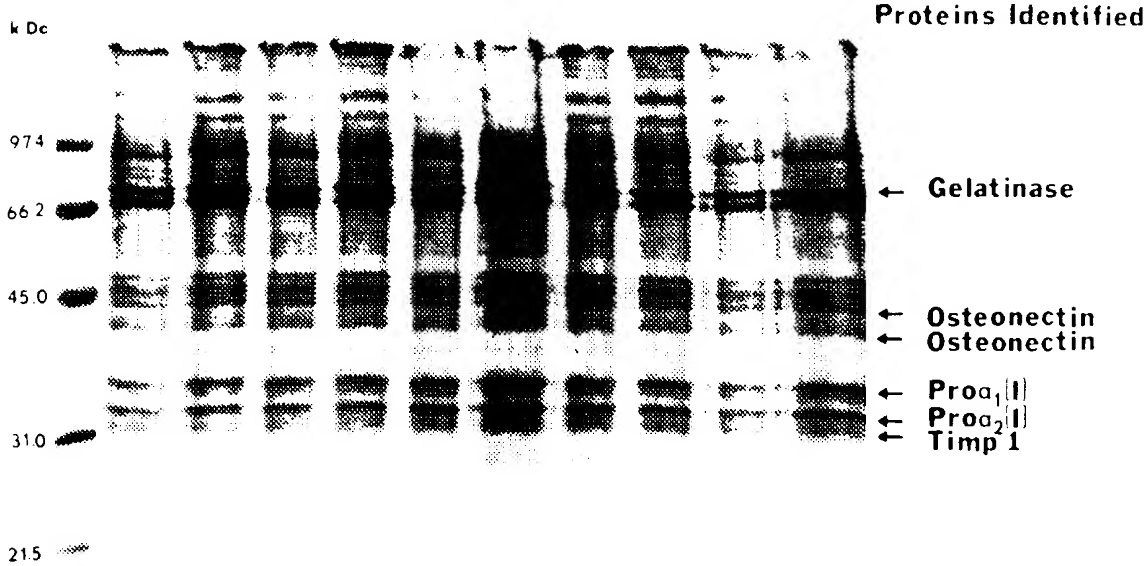

FIG. 2. SDS-polyacrylamide gel analysis of proteins secreted from adult human bone cells. Cells were cultured to confluence in the presence of 10% newborn calf serum and then changed to serum-free medium. Beginning 24 h after the change to serum-free medium, conditioned medium was collected daily for 7 days, dialyzed against H_2O and pooled. Pooled medium (60 ml) was dried and dissolved in 200 μl SDS loading buffer, and 20 μl of this mixture was loaded onto each of 10 lanes of a 10% SDS-polyacrylamide gel. After electrophoresis, proteins were transferred to a PVDF membrane and stained with Coomassie blue. The prominent bands at 72, 67, 49, 44, 41, 35, 33, and 31.5 kD were excised, and the bands at a given M_r from all 10 lanes were subjected to N-terminal sequence analysis (see Materials and Methods).

volume of β-mercaptoethanol and heated for 5 minutes at 100°C to reduce disulfide bonds. After electrophoresis, proteins were electrophoretically transferred from polyacrylamide gels to polyvinylidene fluoride (PVDF) membranes as described by Matsudaira[10] at 90 V for 20 minutes. Protein bands were visualized in gels or on PVDF membranes by staining with Coomassie blue R-250, and protein bands on PVDF membranes were stored at −70°C until further analyzed. To obtain sufficient material for analysis, the same band was excised from up to 10 lanes, each of which contained the protein in up to 6 ml conditioned medium. All 10 bands were placed into the sequencer cartridge and subjected to N-terminal sequence analysis. We typically place the PVDF bands so that they occupy the 1 cm diameter circle of the sequencer cartridge as fully as possible without overlapping membranes and consequently inhibiting solvent flow.

N-terminal sequence analysis

Protein bands were sequenced using an Applied Biosystems 470A sequenator equipped with a model 120 on-line HPLC using the 03RPTH program provided by the manufacturer.[11] Data reduction was accomplished with a Perkin-Elmer 7500 computer equipped with Chrom 3 software. Sequences were searched against the Protein Identification Resource (PIR) protein data base of the National Biomedical Research Foundation, Washington, DC.

Amino acid analysis

Proteins on PVDF membranes were hydrolyzed in 6 N HCl, and the amino acids were extracted with 30% methanol (vol/vol) in 0.1 N HCl as described.[12] Dried hydrolysates were derivatized with 9-fluorenylmethylchloroformate (FMOC)[13,14] and analyzed using a Perkin-Elmer Series 4 HPLC equipped with a Varian Amino Tag C18 column (4.6 mm × 15 cm) and a Kratos Model FS970 fluorescence detector.

Radioimmunoassays

BGP was measured by a specific RIA using an antibody raised against purified bovine BGP.[15] The C-terminal propeptide of type I procollagen (PICP) was measured by a commercially available radioimmunoassay (RIA; PICP RIA Kit, Farmos Diagnostica) using an antibody raised against human PICP purified from normal skin fibroblast cultures.[16] Conditioned medium (100 μl) was used for the

TABLE 1. IDENTIFICATION OF PROMINENT PROTEINS SECRETED BY ADULT HUMAN OSTEOBLASTS[a]

Protein	*N-terminal sequences*															
12 kD band	I	Q	X	(T)	P	(K)	I	Q	V	(Y)	X	X	X	(P)		
β_2-Microglobulin[17]	**I**	**Q**	**R**	**T**	**P**	**K**	**I**	**Q**	**V**	**Y**	**S**	**R**	**H**	**P**		
31.5 kD band	X	T	(C)	V	P	P	X	P	Q	(T)	A	F	X	N	(S)	D
TIMP-1[18]	**C**	**T**	**C**	**V**	**P**	**P**	**H**	**P**	**Q**	**T**	**A**	**F**	**C**	**N**	**S**	**D**
33 kD band	D	Q	P	X	(S)	A	P	(S)	L	X	P	X	(D)	X	(E)	
CO_2H-pro-α_2(I)-collagen[19]	**D**	**Q**	**P**	**R**	**S**	**A**	**P**	**S**	**L**	**R**	**P**	**K**	**D**	**Y**	**E**	
35 kD band	D	D	A	N	V	V	X	D	X	(D)						
CO_2H-pro-α_1(I)-collagen[20]	**D**	**D**	**A**	**N**	**V**	**V**	**R**	**D**	**R**	**D**						
41 kD band	A	P	Q	Q	E	X	L	P	(D)							
Osteonectin[21,22]	**A**	**P**	**Q**	**Q**	**E**	**A**	**L**	**P**	**D**							
44 kD band	(A)	P	Q	X	X	(A)	(L)	(P)	D	(E)	X	(E)	V	V		
Osteonectin[21,22]	**A**	**P**	**Q**	**Q**	**E**	**A**	**L**	**P**	**D**	**E**	**T**	**E**	**V**	**V**		
72 kD band	A	P	S	P	I	(I)	X	F	P	G	D	X	(A)			
Gelatinase[23]	**A**	**P**	**S**	**P**	**I**	**I**	**K**	**F**	**P**	**G**	**D**	**V**	**A**			

[a]The N-terminal sequences of the protein bands described in Fig. 2 are presented, except for the band at 49 kD, which failed to give clear sequence information. The sequence of β_2-microglobulin was obtained from a 15% polyacrylamide gel of the same conditioned medium (data not shown). The reference sequence is shown in boldface type below each sequence obtained; tentative identification in parentheses.

BGP assay and 10 μl for the PICP assay, and the detection limit is 0.2 and 34 ng/ml, respectively.

RESULTS

Isolation and identification of proteins secreted by adult human osteoblastic cells

To better understand the biochemical functions of the osteoblast, we developed a simple and reproducible method for the identification of proteins secreted into serum-free medium by osteoblastic cells in culture. In this procedure, serum-free conditioned medium is collected daily, dialyzed against Milli-Q water, and freeze-dried using either a rotary evaporator or a lyophylizer. Dried protein from up to 6 ml conditioned medium is then loaded onto each lane of an SDS minigel and, after electrophoresis, the separated proteins are transferred to a PVDF membrane and stained with Coomassie brilliant blue. The bands corresponding to the principal proteins are excised, and bands from up to 10 lanes are then loaded onto the sequencer and subjected to N-terminal sequence analysis or to acid hydrolysis and amino acid analysis. To better resolve proteins in given molecular weight ranges, several gels with different percentage cross-linking are used in the analysis of the conditioned medium proteins.

This procedure was used to evaluate the effect of several variables on the pattern of proteins secreted by osteoblastic cells from adult human trabecular bone. Conditioned medium proteins obtained daily for 7 consecutive days of culture in the absence of serum were compared in a single electrophoresis gel to evaluate the possible effect of prolonged culture under serum-free conditions on the pattern of secreted proteins. No significant differences could be detected (data not shown); the gel patterns starting with the first 24 h in serum-free medium are shown here for fetal human osteoblasts and MG-63 osteosarcoma cells (see below). To evaluate the possibility that passage number might affect the pattern of secreted proteins, conditioned media from

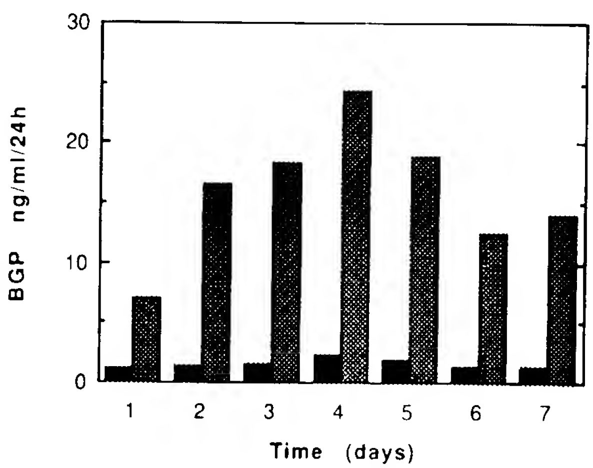

FIG. 3. Rate of BGP secretion in control and 1,25-$(OH)_2D_3$-treated adult human bone cells. Cultures received fresh serum-free medium containing 10 ng/ml of 1,25-$(OH)_2D_3$ (hatched bars) or vehicle (solid bars) every 24 h. The BGP levels in each 24 h conditioned medium were determined by radioimmunoassay of samples in triplicate.

different passages were compared (each passage is the result of a 1:2 subcultivation). No significant differences were seen in conditioned medium from adult human trabecular bone osteoblastic cells between passages 1 and 4 (data not shown), and all results reported here were consequently carried out on conditioned medium from trabecular bone osteoblastic cells at passage 1 or 2. The reproducibility in the pattern of proteins secreted by osteoblastic cells from trabecular bone was evaluated for 10 different adult human donors. The results of this study revealed an essentially identical pattern of proteins in the conditioned medium of each osteoblastic culture (Fig. 1).

A representative Coomassie-stained PVDF membrane from the pooled conditioned medium of osteoblastic cells from passage 2 of trabecular bone donor 5 is shown in Fig. 2. In this example, sufficient protein was present for the identification by N-terminal sequence analysis of gelatinase, osteonectin, TIMP-1, and the C-terminal propeptides of the α_1(I) and α_2(I) chains of collagen (Table 1). The presence of two osteonectin bands with different apparent molecular masses (Table 1) was noted in earlier studies of proteins secreted by human bone cells.[5] Subsequent experiments using a higher protein load per lane and a higher percentage of cross-linked gel identified β_2-microglobulin. One of the other bands tested, at 67 kD (Fig. 2), gave a strong human serum albumin sequence[24] in three conditioned medium samples, which included cells from two different human donors at passage 1 and cells from a third donor at passage 5. For two other conditioned medium samples obtained from an additional two donors, it was not possible to obtain an unambiguous human albumin sequence for the 67 kD band. In addition, northern blot analysis of the mRNA isolated from the cultured osteoblasts from two donors failed to reveal any hybridization when probed with the full-length cDNA for human serum albumin. It seems probable that the human serum albumin sequence obtained for several conditioned medium samples reflects trace contamination of the medium with human serum, although we cannot exclude the possibility that human serum albumin is in fact expressed by some human osteoblast cultures.

To confirm the osteoblastic character of the bone cells used in this study, confluent cultures from each of the 10 trabecular bone donors were assessed for the capacity to increase BGP secretion in response to 10 ng/ml of 1,25-$(OH)_2D_3$. Radioimmunoassay of conditioned medium from the 10 bone cell cultures revealed 12–50 ng/ml of BGP in cells treated for 48 h with 10 ng/ml of 1,25-$(OH)_2D_3$ and undetectable to 2 ng/ml of BGP in the absence of hormone treatment. Similar stimulation of BGP synthesis in adult human bone cells by 1,25-$(OH)_2D_3$ was noted by others.[2,8,25] Daily production of BGP remained

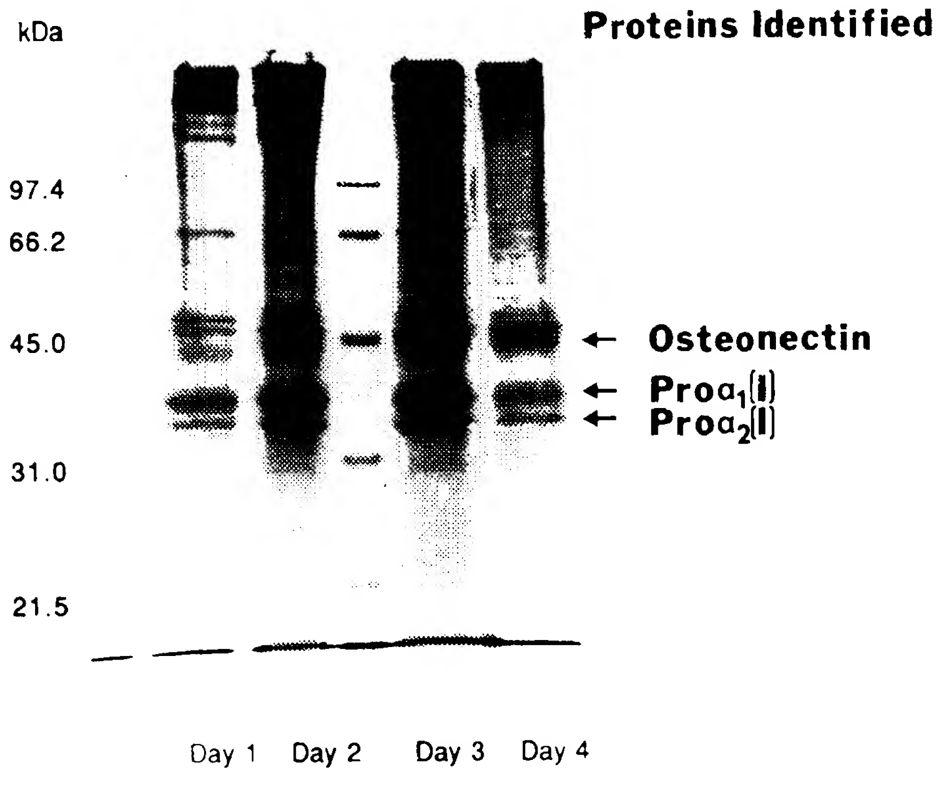

FIG. 4. SDS-polyacrylamide gel analysis of proteins secreted from fetal human bone cells. Cells were cultured to confluence in the presence of 10% newborn calf serum and then changed to serum-free medium. Serum-free conditioned medium was collected daily and dialyzed against H_2O, and 4 ml medium from each indicated day of culture was dried. The dried medium was dissolved in 20 μl SDS loading buffer, loaded onto a lane of a 10% SDS-polyacrylamide gel, electrophoresed, and stained with Coomassie blue (see Materials and Methods).

high over 7 days of 1,25-$(OH)_2D_3$ treatment in serum-free medium for each bone culture tested (results from a representative culture are shown in Fig. 3). Radioimmunoassay of the control medium samples revealed comparably high daily production (8 μg per 10^6 cells per 24 h) of the C-terminal propeptide of type I procollagen over the 7 days in serum-free medium. The adult bone cells were also shown to be osteoblastic by their ability to form a mineralized matrix.[26] The cells were cultured in medium containing 10% serum and supplemented with 10 mM β-glycerophosphate, and medium was changed daily (see Materials and Methods). After 4 weeks, mineralized areas were observed as clear white spots that, when stained with von Kossa stain, became black, indicating that calcium mineral deposition had occurred (data not shown).

Isolation and identification of proteins secreted by fetal human bone cells

The pattern of proteins secreted by fetal human osteoblastic cells, shown in Fig. 4, is markedly different from that obtained for osteoblastic cells from adult trabecular bone (Figs. 1 and 2). The three major Coomassie blue-stained proteins in the conditioned medium of fetal human bone cells were identified by N-terminal sequence analysis to be human osteonectin and the C-terminal propeptides of the α_1(I) and α_2(I) chains of human collagen (Table 2). BGP could not be detected by radioimmunoassay in the culture medium of basal or 1,25-$(OH)_2D_3$-treated fetal human bone cells, and northern blot analysis failed to reveal the presence of BGP mRNA (data not shown).

Isolation and identification of proteins secreted by MG-63 human osteosarcoma cells

Unlike the osteoblastic cells from fetal and adult bone, neither of which reveal significant differences in major Coomassie blue-stained proteins secreted in response to 1,25-$(OH)_2D_3$ treatment or to time in serum-free medium, one component in the medium of the MG-63 human osteosarcoma cell line is induced by 1,25-$(OH)_2D_3$ and by time in culture, the component at an apparent molecular weight of 40 kD (Fig. 5). N-terminal sequencing of this component revealed it to be a new human protein (Table 3), which we termed YKL-40 based on the one-letter code for its first three amino acids and its molecular weight.[30] The N-terminal sequence of YKL-40 has a high degree of identity with the N-terminal sequence of a 39 kD protein isolated from the whey of nonlactating cows[28] and is identical to a protein recently isolated from the conditioned medium of human synovial cells (Table 3).[29] The amino acid composition of YKL-40 is shown in Table 4. Although YKL-40 is the only protein band in the SDS gel shown in Fig. 5 affected significantly by vitamin D treatment or by day of culture, radioimmunoassay of culture medium and northern blot analysis of cellular mRNA revealed that BGP is stimulated by vitamin D in these cultures (data not shown), in agreement with previous results.[1]

Only one additional protein was present in sufficient amounts in the conditioned medium of MG-63 cells for direct identification by the method described here, the 67 kD band (Fig. 5), which proved to be gelatinase. To overcome the limitations imposed by the low level of protein in MG-63 cell conditioned medium, we fractionated the serum-free conditioned medium from MG-63 cells by reversed-phase HPLC, a procedure that effectively concentrated proteins from 3.5 liters conditioned medium, desalted the proteins into a volatile buffer, and partially fractionated the proteins in the mixture. Fractions from the HPLC chromatogram were dried and then further separated by SDS-gel electrophoresis (Fig. 6). Three additional proteins were identified by this procedure, β_2-microglobulin, TIMP-1, and a third protein that was initially found to be related in sequence to TIMP-1 and was later shown to be identical to a subsequently reported human protein, TIMP-2.[27] Despite this medium concentration and partial purification step, no bands could be detected at the molecular weight positions expected for osteonectin and for the C-terminal propeptides of type I collagen, which indicates that MG-63 cells produce very little of these extracellular matrix constituents.

TABLE 2. IDENTIFICATION OF PROMINENT PROTEINS SECRETED BY FETAL HUMAN OSTEOBLASTS[a]

Protein	*N-terminal sequences*
33 kD band	D Q P R S A P S L (R) P K D Y E V D A T L
CO_2H-pro-α_2(I)-collagen[19]	**D Q P R S A P S L R P K D Y E V D A T L**
35 kD band	D D A N V V (R) D X D L E V D T X L K X L X Q (Q)
CO_2H-pro-α_1(I)-collagen[20]	**D D A N V V R D R D L E V D T T L K S L S Q Q**
44 kD band	A P Q Q E A L P D E T E V X E
Osteonectin[21,22]	**A P Q Q E A L P D E T E V V E**

[a]Cells were grown to confluence in the presence of 10% newborn calf serum and then changed to serum-free medium. Beginning 24 h after the change to serum-free medium, conditioned medium was collected daily for 7 days, dialyzed against H_2O, and pooled. Pooled medium (60 ml) was dried and dissolved in 200 μl SDS loading buffer, and 20 μl of this mixture was loaded onto 10 lanes of a 10% SDS–polyacrylamide gel. After electrophoresis, proteins were transferred to a PVDF membrane and stained with Coomassie blue (data not shown). The prominent bands at 44, 35, and 33 kD (Fig. 4) were excised, and the bands from all 10 lanes were subjected to N-terminal sequence analysis (see Materials and Methods). The reference sequence is shown in boldface type below each sequence obtained; tentative identification in parentheses.

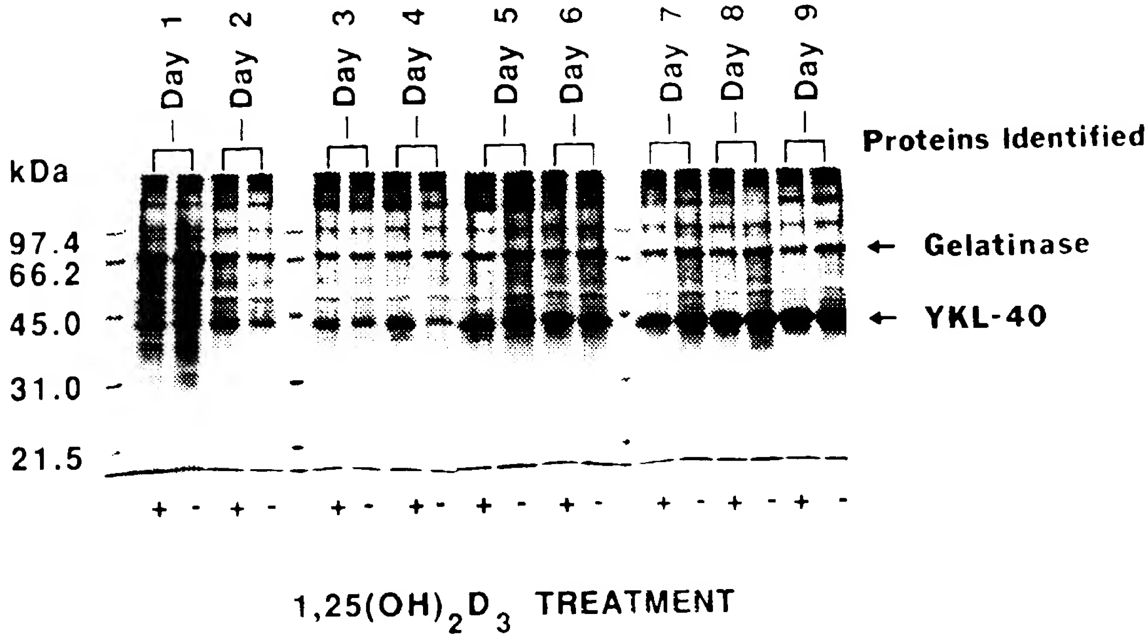

FIG. 5. SDS-polyacrylamide gel analysis of proteins secreted from MG-63 cells in response to 1,25-$(OH)_2D_3$ treatment. MG-63 cells were grown to confluence in the presence of 10% newborn calf serum and then changed to serum-free medium containing either 10 ng/ml of 1,25-$(OH)_2D_3$ (+) or an equal volume of vehicle (−). Medium was collected daily and replenished with fresh media. The daily medium collections were dialyzed against H_2O and 4 ml medium from each indicated day was dried. The dried medium was dissolved in 20 μl SDS loading buffer, loaded onto a 10% SDS-polyacrylamide gel, electrophoresed, and stained with Coomassie blue (see Materials and Methods).

DISCUSSION

The procedure developed in the present study for the identification of proteins secreted by cells in culture has the advantages that all secreted proteins can in principle be identified and the relative abundance of the proteins can be quantitatively determined. The method is also highly reproducible, allowing the changes in protein secretion due to such variables as hormonal treatment or cell type to be detected by direct comparison of stained protein bands, which can be subsequently identified by N-terminal sequence analysis (see Fig. 5). The major limitation of this procedure appears to be the amount of protein required for N-terminal sequence analysis. We identified proteins by N-terminal sequence analysis with an initial yield as low as 3 pmol. Based on amino acid analysis of PVDF bands, this initial yield is typically 10–40% of that expected from the amount of protein present; this initial yield is not atypical for proteins transferred to PVDF membranes.[31] To identify an average protein of 30 kD, at least 1 μg total protein must therefore be present in the up to 10 bands used for sequence analysis. The limitations caused by the low levels of protein in a band can be overcome in some instances by a preliminary purification step, such as the separation of conditioned medium proteins by reversed-phase chromatography before gel electrophoresis (e.g., Fig. 6). This preliminary purification step, however, was unnecessary for the identification of many of the most abundant proteins in the conditioned medium of the osteoblastic cells studied here.

The major focus of our studies was the identification of proteins under 100 kD secreted by normal human osteoblasts from trabecular bone. This investigation revealed that osteoblastic cells from 10 different adult human donors secrete a pattern of proteins that is essentially identical in the number of prominent components under 100 kD that can be visualized by Coomassie staining and in the relative amounts of these components. These observations, together with the consistently high production of the bone-specific BGP in response to 1,25-$(OH)_2D_3$ in each culture, strongly indicate that the cell populations studied here are uniform in phenotype over the first several passages tested. Since the pattern of proteins secreted by any given adult human osteoblast culture did not change over 7 consecutive days of culture, it is clear that the pattern of proteins secreted by these cells is not affected by prolonged culture under serum-free conditions. It is important to note, however, that the initial transition to serum-free conditions could have produced a rapid change in the expression of some secreted proteins that cannot be detected by comparison of the first 24 h conditioned medium sample with subsequent days of medium collection. In addition, the conditions osteoblasts encounter in cell culture are far from those they typically encounter in vivo, and therefore the re-

TABLE 3. IDENTIFICATION OF PROMINENT PROTEINS SECRETED BY MG-63 OSTEOSARCOMA CELLS[a]

Protein	*N-terminal sequences*
12 kD band	I Q X T P K I Q V Y S R H P A E N G K S N
β_2-Microglobulin[17]	**I Q R T P K I Q V Y S R H P A E N G K S N**
25 kD band	X (S) X S P V (H) P Q Q A F (C) N A D V (V) I (R) A K A V X E K (E) (V)
TIMP-2[27]	**C S C S P V H P Q Q A F C N A D V V I R A K A V S E K E V**
31 kD band	X X X V P P H P Q T A F X N S D L V I X A K F V
TIMP-1[18]	**C T C V P P H P Q T A F C N S D L V I R A K F V**
40 kD band	Y K L V (S) Y Y T S X S Q Y R E G D X S X (F) (P) (K)
Bovine whey protein[28]	**Y K L I X Y Y T S W S Q Y R E G D G S X F P D**
Human synovial cell protein[29]	**Y K L V C Y Y T S W S Q Y R E G D G S X F P D**
69 kD band	(A) P S P I I K F P G D V A P K (T) X (K)
Gelatinase[23]	**A P S P I I K F P G D V A P K T D K**

[a]The 40 and 69 kD bands were isolated for N-terminal sequence analysis by electrophoresis of conditioned medium using procedures described in Fig. 2. Bands were also isolated for sequence analysis by C4 HPLC followed by electrophoresis. The 12 kD band was obtained from electrophoresis of HPLC fraction 84, the 25 and 31 kD bands from HPLC fraction 86, the 40 kD band from HPLC fraction 98, and the 69 kD band from HPLC fraction 96 (see Fig. 6). The two methods of isolating the 40 and 69 kD components yielded essentially identical sequence data. The reference sequence is shown in boldface type below each sequence obtained; tentative identification in parentheses.

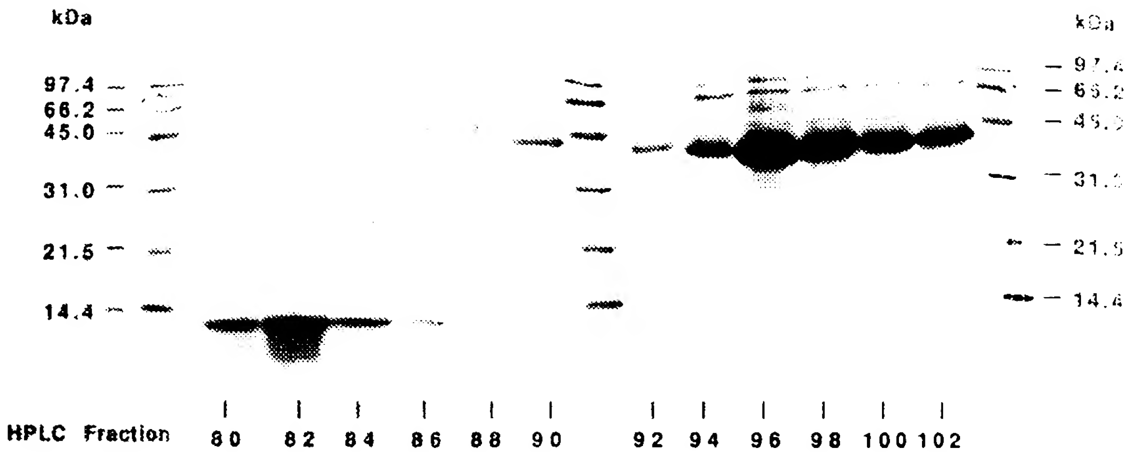

FIG. 6. SDS–polyacrylamide gel analysis of fractions from the C4 reversed-phase HPLC of MG-63 conditioned medium. Aliquots (200 μl) from every other fraction of the preparative C4 reversed-phase chromatography of 3.5 liters MG-63 medium were dried, dissolved in 20 μl SDS loading buffer, and electrophoresed on a 15% SDS-polyacrylamide gel (see Materials and Methods).

lationship between the present results and the pattern of protein expression in situ remains to be established.

Some of the major Coomassie blue-staining constituents in the conditioned medium of adult bone cells correspond to proteins that are prominent constituents of bone matrix, and their abundance in the medium of these bone-forming cells is not unexpected. These proteins include the C-terminal propeptides of type I collagen and osteonectin. Other comparably prominent constituents of osteoblast conditioned medium, gelatinase, TIMP-1, and β_2-microglobulin, are not major constituents of the matrix of bone and presumably play an as yet uncharacterized role in matrix turnover or formation. The present study is the first to show that, under cell culture conditions, these nonmatrix proteins are secreted at a rate comparable to matrix constituents, a result that confirms the importance of the study of conditioned medium as an adjunct to the analysis of bone matrix itself in the study of bone metabolism. One protein we expected to find in conditioned medium, the N-terminal propeptide of type I collagen, was not found in the 25 kD position in quantities sufficient for N-terminal sequence analysis. Since quite high levels of the C-terminal propeptides were present, the N-terminal propeptide either is generated from procollagen more slowly than the C-terminal propeptide under culture conditions or is selectively lost from conditioned medium during culture or purification.

The present investigations confirm previous studies that showed the apparent absence of BGP expression in osteoblastic cells from fetal bone[32–34] and also demonstrate other marked differences between the fetal and adult cell. Of particular note is the prominence of proteins in medium that correspond to constituents of the extracellular matrix, namely the C-terminal propeptides of type I collagen and osteonectin, and the apparent absence of proteins that are not also matrix constituents, gelatinase and TIMP-1. These observations suggest that fetal bone cells may primarily express constituents of bone matrix and lack prominent expression of proteins that could be involved in other aspects of bone metabolism and indicate that fetal cells are not a good model for bone metabolism in the adult.

Although the MG-63 osteosarcoma cell line has been used widely as a model for the human osteoblast, the present results demonstrate dramatic differences in Coomassie blue-staining secreted proteins between MG-63 cells and adult osteoblasts. Of particular note is the apparent absence of proteins in the medium of MG-63 cells that correspond to prominent constituents of the extracellular bone matrix. For example, C-terminal collagen propeptides cannot be detected in the medium of these cells (Figs. 5 and 6). These observations indicate that MG-63 cells may not be a good model for matrix formation in the adult human but, rather, could reflect a more specialized bone cell, such as the osteocyte, which is less involved in matrix formation than in matrix maintenance and turnover.

One of the major strengths of the methods described here for the analysis of matrix-forming cells is that prominent secreted proteins can be identified that would normally be overlooked because they are not prominent matrix constituents, such as YKL-40. This protein was identified as a secreted protein of human bone cells in vitro for the first time in the present study. YKL-40 may be viewed as a protein that, based on its singular induction by 1,25-$(OH)_2D_3$ among the media proteins of MG-63 cells, could play a role in the action of vitamin D in bone. We anticipate that future improvements in sequencing sensitivity and applications of the methods described here to other cells will further broaden our understanding of the metabolism of bone and other tissues.

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Downregulation of SPARC Expression Is Mediated by Nitric Oxide in Rat Mesangial Cells and during Endotoxemia in the Rat

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Abstract. Nitric oxide (NO) has been implicated in several forms of glomerulonephritis. In this study, a low stringency reversed transcription/PCR protocol was used to evaluate the action of NO on the mRNA expression pattern in rat mesangial cells (MC). To mimic the state of glomerular inflammation, MC were stimulated by exposure to the cytokines interleukin-1β and tumor necrosis factor-α into producing high levels of NO via expression of inducible nitric oxide synthase (NOS). To detect NO-mediated effects, the resulting expression pattern was compared to that of MC stimulated by the cytokines in the presence of the NOS inhibitor N^G-monomethyl-L-arginine (L-NMMA). Computer analysis of a differentially expressed cDNA fragment resulted in a 100% homology to the recently characterized mRNA of SPARC (secreted protein acidic and rich in cysteine). Further characterization of SPARC regulation revealed a cytokine- and cAMP-dependent decrease in SPARC mRNA and protein levels. Blocking NO formation by L-NMMA reversed the effects of cytokines and cAMP on SPARC expression, suggesting an NO-mediated mechanism. The NO donors *S*-nitroso-*N*-acetyl-penicillamine and diethylenetriamine/NO further reduced SPARC expression in cytokine-treated MC as well as in controls. Moreover, downregulation of SPARC mRNA and protein expression in whole kidneys obtained from rats treated with endotoxin was observed. This downregulation of SPARC was reversed by treatment with L-N^6-l (iminoethyl) lysine dihydrochloride, a potent and highly selective inhibitor of inducible NOS. These data characterize SPARC as an NO-regulated gene. This observation may be important in the context of tissue remodeling in chronic inflammatory kidney diseases.

Inflammatory diseases of the renal glomerulus are associated with an enhanced formation of the radical nitric oxide (NO) (1–4). The rat mesangial cell (MC), a smooth muscle cell-like pericyte located in the glomerulus, has been suggested to function as a key player of the glomerular inflammatory response (5). Primary cultures of MC react to the cytokines interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) or with membrane-permeable cAMP analogs leading to the generation of NO secondary to the induction of the inducible form of NO synthase (iNOS) (6,7). Besides its ability to induce apoptosis and cell death in glomerular mesangial cells (8,9), NO also mediates the expression of certain genes on the transcriptional level. NO directly modulates gene transcription of iNOS (10) and macrophage inflammatory protein 1α (MIP-1α) (11). Recently, new technologies, most of which are based on PCR, have been developed to characterize changes in the mRNA expression patterns in various cell culture or tissue systems. Here, we use a modified version of a low-stringency PCR method originally developed by Welsh *et al.* (12) and now available by Stratagene (RNA arbitrarily primed PCR [RAP-PCR]) to study the action of NO on the gene transcription pattern of MC. To detect genes that undergo NO-mediated regulation, we compared the expression patterns of MC stimulated into producing high, but subtoxic, amounts of NO using a combination of the cytokines IL-1β and TNF-α. In addition, MC were treated with the potent NOS-inhibitor N^G-monomethyl-L-arginine (L-NMMA). Using this approach, we found that NO produced an inhibitory effect on the expression of SPARC (secreted protein acidic and rich in cysteine, also known as BM-40 or osteonectin). The highly glycosylated SPARC protein shows a variety of biologic activities (13), and its action as a scavenger of platelet-derived growth factor (PDGF) may be of relevance in the course of glomerulonephritis as shown recently by Pichler *et al.* (14). By modulating SPARC expression, NO may subsequently affect MC proliferation in the course of glomerular inflammation.

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Materials and Methods

Reagents

Human recombinant IL-1β was kindly provided by Dr. Ch. Rordorf (Novartis Pharma, Inc., Basel, Switzerland). TNF-α was a gift from Knoll AG (Ludwigshafen, Germany). The anti-SPARC antibody was a generous gift from Dr. R. Timpl (Martinsried, Germany). A cDNA clone representing the murine iNOS (pMac-NOS) was generously provided by Dr. J. Cunningham (Boston, MA). α-^{32}P-dCTP, α-^{33}P-dCTP, and "ready prime" DNA labeling kit were obtained from Amersham Buchler (Braunschweig, Germany). Nylon blotting membranes were from Life Science (Schleicher & Schuell, Dassel, Germany) or Millipore (Eschborn, Germany). Tissue culture plastic was from Falcon (Becton-Dickinson, Heidelberg, Germany), and media and sera were from Life Technologies (Eggenstein, Germany). NO donors, L-NMMA and L-N^6-1 (iminoethyl) lysine dihydrochloride (L-NIL) were from Alexis (Grünberg, Germany). Chemicals for reverse transcription (RT)-PCR were obtained from Stratagene (Amsterdam, The Netherlands). Sodium thiopentone (Intraval Sodium) was obtained from Rhone Merieux Ltd. (Harlow, Essex, United Kingdom). Bacterial lipopolysaccharide (LPS) (*Escherichia coli* serotype 0.127:B8) was from Sigma (Poole, Dorset, United Kingdom). All other chemicals were purchased from Sigma (Deisenhofen, Germany).

Cell Culture and Stimulation

Rat glomerular MC were cultured as described previously (15). MC were grown in RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum, 2 mM glutamine, 5 ng/ml insulin, 100 U/ml penicillin, and 100 μg/ml streptomycin. To obtain quiescent cells, MC were maintained in serum free Dulbecco's minimal essential medium supplemented with 0.1 mg/ml fatty acid-free bovine serum albumin for 24 h before cytokine or cAMP treatment (IL-1β, 2 nM; TNF-α, 2 nM; Bt_2cAMP, 1 or 5 mM in combination with IL-1β). MC were used between passages 8 and 19. Unless indicated otherwise, NOS inhibitors were administered at a concentration of 2 mM. Viability of MC was not altered under the conditions used for the experiments described as determined by lactate dehydrogenase measurement in the culture medium (cytotoxicity detection kit; Boehringer Mannheim, Mannheim, Germany).

RNA Arbitrarily Primed PCR

mRNA from MC was prepared by use of an mRNA isolation kit (Stratagene), and low-stringency RT-PCR was performed according to the instructions provided with the RAP-PCR kit (Stratagene). As an internal control, the primers provided with the kit were exchanged for the following primers designed for rat iNOS cDNA:

N3: 5′-CC**GGATCC**TCTTTGCTACTGAGACAGG-3′

N4: 5′-CC**GAATTC**GGGATCTGAATGCAATGTT-3′

Briefly, 100 ng of mRNA obtained from differently treated MC were reverse-transcribed by use of primer N4 that matches to the published rat cDNA at position 2098–2077 (16) (accession no. D14051). Low-stringency PCR was performed in the presence of both N4 and N3 (position 1634–1654) and α-^{33}P-dCTP for one cycle at an annealing temperature of 35°C. Cycling was then continued for an additional 40 cycles at 53°C. The radiolabeled cDNA fragments were separated on a sequencing gel and exposed to x-ray film. Bands that obviously represented NO-regulated cDNA were excised and reamplified under stringent PCR conditions as mentioned above. The intrinsic *Bam*HI and *Eco*RI restriction sites (in boldface) in N3 and N4, respectively, allowed easy cloning of the PCR products into the respective sites of the vector Bluescript KS$^+$ (Stratagene). Sequencing of the PCR products was performed using an automated sequence analyzer A310 (Perkin Elmer Applied Biosystems, Weiterstadt, Germany).

Nitrite Analysis

Nitrite (NO_2^-), the stable end product of NO, was measured in the culture medium by the Griess method (17) using a "ready to use" Griess reagent (Merck, Darmstadt, Germany).

Northern Blot Analysis

Isolation of total RNA from MC and Northern blotting were performed as described previously (18,19). Filter-bound RNA was hybridized to the radiolabeled *Sma*I fragment of the murine cDNA clone pMac-NOS or a 680-bp *Bam*HI/*Eco*RI fragment obtained by RAP-PCR, which represented SPARC cDNA. Equivalent loading of the RNA probes was corrected after rehybridization of the filter with a cDNA probe for GAPDH. The mRNA levels for SPARC, iNOS, and GAPDH were measured by an automated detector system BAS 1500 of Fuji film (Raytest, Straubenhardt, Germany). iNOS and SPARC mRNA levels were corrected for by the mRNA levels of GAPDH and are expressed as arbitrary units.

Western Blot Analysis

MC cultured in 10-cm dishes were lysed in 20 mM Tris-HCl, pH 7.5, 1 mM ethylenediaminetetra-acetic acid, 1 mM ethyleneglycol-bis(β-aminoethyl ether)-*N,N′*-tetra-acetic acid, 1% Triton X-100, 2 mM dithiothreitol, 50 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride and left on ice for 15 min. Extracts were sonicated three times for 10 s and centrifuged at 13,000 rpm for 2 min. The protein concentration of the lysate was determined by the Bradford protein assay (Bio-Rad, Munich, Germany). A total of 100 μg of total protein from each sample was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (15% [wt/vol] for SPARC, 8% [wt/vol] for iNOS). Subsequently, the protein was blotted to a nylon membrane and immunoreactive protein was detected using an anti-iNOS N-terminal antibody (20) or with anti-SPARC antibody raised against murine recombinant SPARC (21). Intensity of the bands representing iNOS (130 kD) or SPARC (43 kD) protein was evaluated using a GS-700 imaging densitometer (Bio-Rad, Munich, Germany).

Rat Model of Endotoxemia

Surgical Procedure. This study was carried out using 48 male Wistar rats (Tuck, Rayleigh, Essex, United Kingdom) weighing 240 to 320 g and receiving a standard diet and water *ad libitum*. The investigation was performed in accordance with the Home Office Guidance on the Operation of the Animals (Scientific Procedures) Act 1986, published by HMSO, London, United Kingdom. All animals were anesthetized using thiopentone sodium (Intraval Sodium, 120 mg/kg intraperitoneally), and anesthesia was maintained by supplementary injections of thiopentone sodium (approximately 1 to 2 mg/kg per h intravenously as required). The trachea was cannulated to facilitate respiration, and rectal temperature was maintained at 37°C using a homeothermic blanket (BioScience, Sheerness, Kent, United Kingdom). The left carotid artery was cannulated and connected to a pressure transducer (Senso-Nor 840; Senso Nor, Horten, Norway) for the measurement of phasic and mean arterial BP (MAP) and heart rate, which were displayed on a data acquisition system (MacLab 8e; AD Instruments, Hastings, United Kingdom) installed on an Apple Macintosh computer. The femoral vein was cannulated for the administration of drugs. The bladder was also cannulated to collect urine.

Upon completion of the surgical procedure, cardiovascular parameters were allowed to stabilize for 15 min.

Experimental Design. After recording baseline hemodynamic parameters, one group of animals received *Escherichia coli* LPS (10 mg/kg intravenously, $n = 36$) as a slow injection over 15 min, after which saline was administered as a continuous infusion (6 ml/kg per h intravenously). Another group of animals was administered LPS as described above, but also the selective iNOS inhibitor L-NIL (22) as an intravenous bolus of 3 mg/kg followed by an infusion of 3 mg/kg per h ($n = 18$). The dose of L-NIL used here is sufficient to abolish the rise in nitrite and nitrate caused by LPS (within 6 h) in the rat (23). Animals subjected to one of the above treatment regimens were killed by an overdose of anesthetic at 30 min and 1, 2, or 4 h after injection of LPS. Subsequently, the right kidney was removed, snap-frozen in liquid nitrogen, and stored at −70°C until use for protein isolation.

Statistical Analyses

Unless otherwise indicated, results represent one of three experiments giving similar results. Data are expressed as percentage *versus* controls (mean ± SD) or as mmHg for MAP (mean ± SD) or beats/min (mean ± SD) for heart rate. Significance was tested by *t* test or one-way ANOVA followed by Bonferroni post-significance testing, and P values <0.05 were considered statistically significant.

Results

For RAP-PCR, mRNA expression in MC in which NO production was stimulated using a combination of IL-1β and TNF-α was compared with mRNA expression in MC exposed to cytokines in the presence of L-NMMA for 24 h as well as mRNA from vehicle-treated control cells. Cytokine-dependent NO production was monitored by measurement of nitrite content in the culture medium. (IL-1β + TNF-α, 81.09 ± 9.1 nmol/mg protein; control, 12.76 ± 0.77 nmol/mg protein (mean ± SD); $P < 0.05$). Nitrite production was inhibited to control levels when L-NMMA was coadministered with the cytokine mix (IL-1β + TNF-α + L-NMMA, 13.69 ± 2.48 nmol/mg protein). In the RAP-PCR-pattern obtained using primers N3 and N4 that have a 100% homology to the iNOS-cDNA over a length of 21 bp, approximately 30 cDNA fragments between 400 and 1000 bp in size were detected (data not shown). As expected, the most prominent band of 464 bp represented iNOS-cDNA as verified by sequencing. This band was not amplified from cDNA of control MC but was clearly visible when cDNA from cytokine-treated MC was used a template. Furthermore, in accordance with data from Mühl and Pfeilschifter (10), who described a potentiation of iNOS expression by NO in MC, the signal was clearly diminished when NO production of cytokine-treated MC was inhibited by the NOS inhibitor L-NMMA (Figure 1A, bottom panel). Compared with the band representing iNOS-cDNA, a band of approximately 680 bp showed an opposite pattern. The signal visible in controls disappeared on cytokine treatment and was restored when NO production was inhibited (Figure 1A, top panel). The DNA band was excised, reamplified, cloned, and sequenced. Using computer-aided search tools in the DDBJ/EMBL/GenBank databases, the DNA fragment was identified as the SPARC cDNA recently cloned by Lee *et al.* (unpublished data) (accession no. D28875) and Liao *et al.* (24) (accession no. Y13714). The NO-dependent regulation of SPARC and iNOS mRNA was verified using Northern blotting of total RNA of MC treated with cytokines or cytokines + L-NMMA in experiments that were performed independently from those used to obtain mRNA for RAP-PCR (Figure 1B, top panel). The expression patterns for iNOS and SPARC did not differ from those obtained in controls when L-NMMA was administered alone to MC without cytokine treatment (data not shown). The same blots, hybridized against an iNOS probe, resulted in the pattern expected for cytokines and NO-dependent iNOS expression (10), confirming the opposite effects of NO on iNOS and SPARC mRNA expression (Figure 1B, middle panel). In further experiments we used the specific iNOS inhibitor L-NIL instead of L-NMMA and observed a comparable reversal of SPARC downregulation by cytokines (Figure 1C).

MC were treated with IL-1β + TNF-α for different time periods to examine whether the effect of NO on SPARC mRNA levels correlated with the time course of iNOS expression. Reduction of SPARC mRNA steady-state levels was significant after 8 h (77.6%) and was more pronounced at 24 h (37.9%) (Figure 2). This downregulation was accompanied by an increase of iNOS mRNA and formation of nitrite measured in the culture medium (data not shown). Further experiments showed that the amount of NO produced by MC treated with IL-1β alone was sufficient to trigger downregulation of SPARC mRNA levels (Figure 3, lane 3). Therefore, TNF-α was omitted as a coinducer of iNOS during the following experiments.

To address the question of whether NO-dependent reduction of SPARC mRNA levels is followed by a downregulation of SPARC protein, immunoblot analysis of SPARC protein levels was performed. MC were treated with IL-1β or IL-1β + L-NMMA for 24 h, and protein extracts were used for Western blot analysis. To determine whether exogenously added NO also triggers SPARC expression, MC were also treated with the NO donor *S*-nitroso-*N*-acetyl-D,L-penicillamine (500 μM) alone or in combination with IL-1β (Figure 4). iNOS protein expression was comparable to data published by Muhl and Pfeilschifter (10), who demonstrated an augmentation of cytokine-induced iNOS expression by NO (Figure 4). SPARC protein expression was diminished to 57.4% by IL-1β. This effect was reversed by L-NMMA to 97.7%, suggesting that downregulation of SPARC protein by IL-1β occurs in an NO-dependent manner (Figure 4). Furthermore, the NO donor *S*-nitroso-*N*-acetyl-D,L-penicillamine diminished SPARC protein levels to 54.4% when compared to control cells and further reduced SPARC protein levels to 25.9% when administered in combination with IL-1β (Figure 4).

In previous studies, we have shown that cAMP acts as a strong inducer of iNOS expression in MC (7,25). Therefore, we also examined the effects of the membrane-permeable cAMP analogue N^6,*O*-2′dibutyryladenosine 3′,5′-phosphate (Bt_2cAMP) on SPARC expression in the absence or presence of increasing concentrations of the NOS inhibitor L-NMMA for 24 h. In a manner comparable to IL-1β, Bt_2cAMP (5 mM) reduced SPARC mRNA levels by 59% when compared with untreated control cells (Figure 3). This effect was reversed by

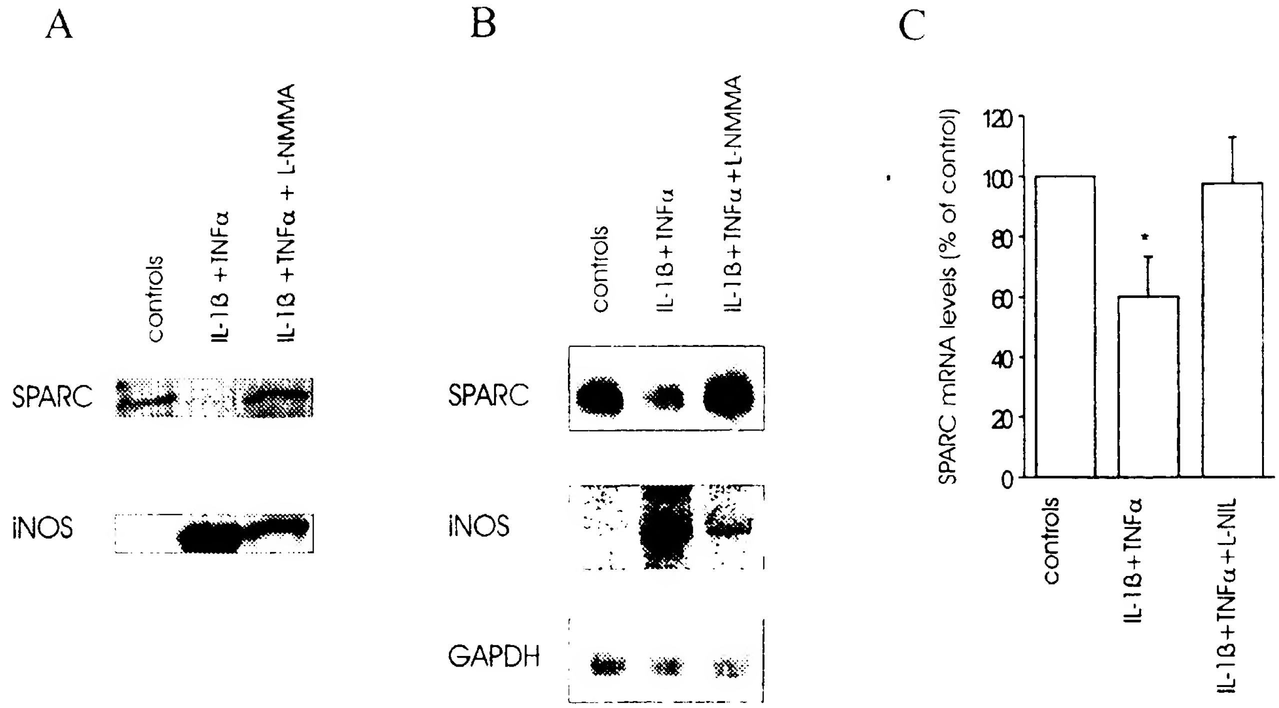

Figure 1. Detection of cytokine-induced downregulation of SPARC (secreted protein acidic and rich in cysteine) by RNA arbitrarily primed PCR (RAP-PCR) and Northern blot. Radiolabeled transcripts obtained from RAP-PCR were separated on a 6% sequencing gel. The parts of the autoradiogram, which represent transcripts of inducible nitric oxide synthase (iNOS) (464 bp) and SPARC (680 bp) from mesangial cells (MC) treated as indicated are shown in Panel A. Verification of SPARC regulation in MC by Northern blot using the cloned and radiolabeled SPARC fragment as well as the iNOS fragment of pMAC NOS is shown in Panel B. Equivalent loading of the RNA probes was corrected after rehybridization of the filter with a probe for GAPDH. The effects of the specific iNOS inhibitor L-N^6-l (iminoethyl) lysine dihydrochloride (L-NIL) on cytokine-induced SPARC mRNA levels is shown in Panel C. mRNA levels were expressed as relative units (SPARC/GAPDH) controls = 100% (n = 3) $*P < 0.05$ *versus* control and interleukin-1β (IL-1β) + tumor necrosis factor-α (TNF-α) + N^G-monomethyl-L-arginine (L-NMMA).

L-NMMA in a dose-dependent manner with complete restoration of SPARC mRNA levels observed using 3 mM L-NMMA, a concentration that completely inhibited NO formation (data not shown). Coincubation of MC with IL-1β and Bt_2cAMP (1 mM) resulted in a threefold amplification of nitrite production compared to cells treated with Bt_2cAMP alone (Figure 3). Concomitantly, a more pronounced downregulation of SPARC mRNA was observed (21.8%), which was partially reversed in the presence of 3 mM L-NMMA to 74%. SPARC protein levels were also diminished by Bt_2cAMP in an NO-dependent manner (Figure 5).

To test whether the downregulation of SPARC is mediated by a direct action of NO or by activation of guanylyl cyclase, MC were treated for 24 h with different concentrations of the NO donor DETA-NO ((Z)-1-[2-(2-aminoethyl)-*N*-(2 ammonioethyl)amino]diazen-1-ium-1,2-diolate), as well as with the membrane-soluble cGMP derivative 8-bromo-cGMP. A concentration-dependent downregulation of SPARC mRNA in DETA-NO-treated MC was observed (Figure 6A). In contrast, even high amounts of 8-bromo-cGMP (1 mM) had no effect on SPARC mRNA expression, suggesting that the regulation of SPARC occurs independently of NO-mediated activation of guanylyl cyclase (Figure 6C).

To evaluate whether our observations from cell culture experiments could be confirmed *in vivo*, rats were treated for different time periods with bacterial LPS, a potent inducer of endogenous NO formation (26). In another group of animals, NO formation was blocked by L-NIL, a highly selective inhibitor of iNOS activity (22). Infusion of LPS (10 mg/kg intravenously) produced a significant fall in mean arterial BP (Table 1). However, treatment of LPS rats with L-NIL significantly attenuated the hypotension caused by LPS after 4 h (Table 1). Treatment of rats with either LPS alone or with LPS plus L-NIL did not have a significant effect on heart rate (Table 1).

Subsequently, the levels of iNOS and SPARC protein from whole kidneys were analyzed using Western blotting. iNOS protein was detectable after 2 h of LPS treatment, and the induction of iNOS was paralleled by a decrease in SPARC protein levels, whereas SPARC protein levels were unchanged in kidneys from LPS-rats treated additionally with L-NIL (Figure 7).

Discussion

NO has been shown to play a key role during the development of several forms of glomerulonephritis (1–4,27). In addition to a wide variety of direct biologic activities of NO

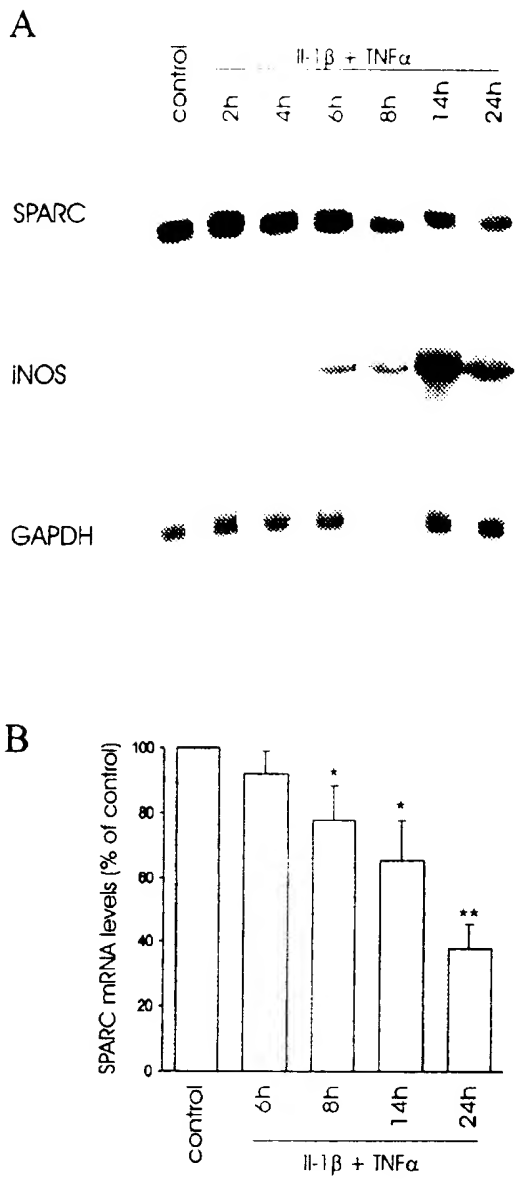

Figure 2. Time course of cytokine-mediated SPARC and iNOS expression. MC were maintained serum-free for 24 h and then treated for different time points as indicated. Total RNA was subjected to Northern blotting against cDNA probes for iNOS and SPARC. Equivalent loading of the RNA probes was corrected after rehybridization of the filter with a probe for GAPDH. iNOS mRNA levels were detected, visualized (A), and evaluated (B) using the automated detector system BAS 1500 of Fuji film (Raytest, Straubenhardt, Germany). mRNA levels for SPARC were corrected with mRNA levels for GAPDH and expressed as percentage of controls (B). The data shown in Panel B represent means ± SD of three independent experiments. $*P < 0.05$ *versus* control; $**P < 0.01$ *versus* control

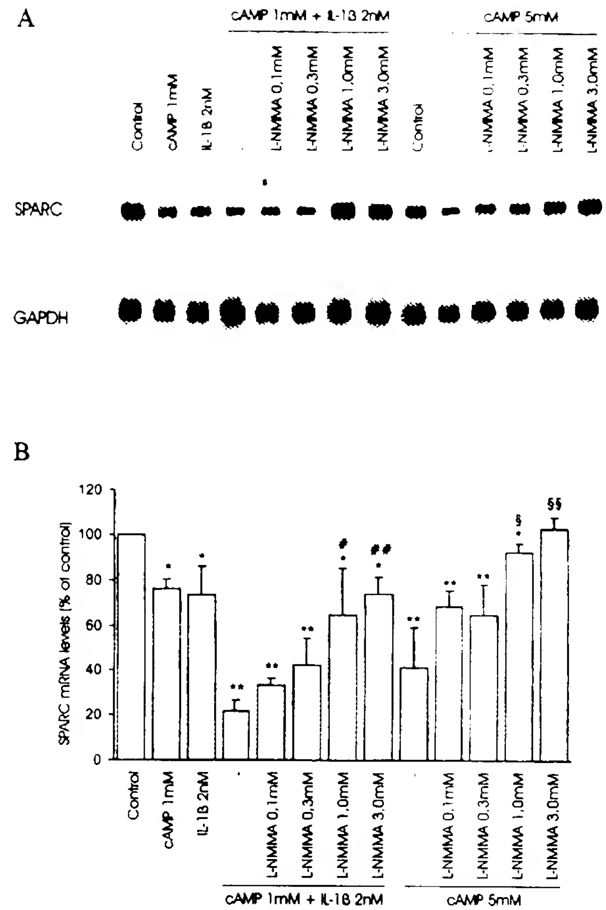

Figure 3. Effects of cAMP-induced NO generation on SPARC mRNA steady-state levels. Quiescent MC were treated as indicated for 24 h. Total RNA was electrophoresed in an agarose gel and blotted onto a nylon membrane. The blot was hybridized against radiolabeled SPARC cDNA. Equivalent loading was determined by rehybridization against GAPDH. Visualized data (one of three independent experiments) are shown in Panel A. Computer-based evaluation of the intensities of SPARC *versus* GAPDH of three independent experiments is shown in Panel B. $*P < 0.05$ *versus* control; $**P < 0.01$ *versus* control; $^{\#}P < 0.05$ *versus cAMP + IL-1β alone;* $^{\#\#}P < 0.01$ versus cAMP + IL-1β alone; $^{§}P < 0.05$ *versus cAMP alone;* $^{§§}P < 0.01$ versus cAMP alone.

(28–30), there is increasing evidence that NO can also act as an important mediator of gene expression (10,11,29,31–35). Therefore, we propose that in MC, NO participates in the pathologic changes in the mRNA expression of several genes during glomerulonephritis. To study the effects of endogenously produced NO in cultured MC, we used a modified, low-stringency RT-PCR protocol originally described as RAP-PCR and compared the gene transcription patterns of cytokine-exposed MC in the presence or absence of the NOS inhibitor L-NMMA. Our modification was related to the use of gene-specific primers. The specific cDNA fragment serves as a size marker and as a standard for comparison of different expression patterns. Thus far, we have used five different gene-specific primers that amplify cDNA for rat iNOS, GAPDH, xanthine oxidase, vascular endothelial growth factor, and basic fibroblast growth factor under stringent conditions. Twelve cDNA that obviously reflect NO-dependent gene expression were further examined. On the level of mRNA expression, NO-dependent regulation of seven genes that show homology

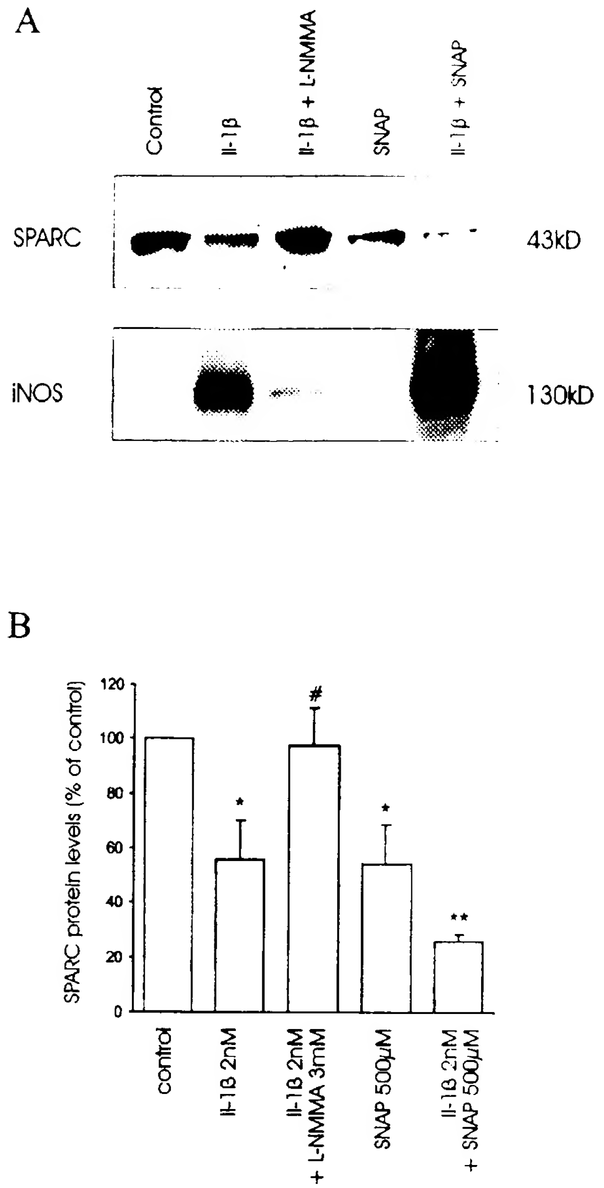

Figure 4. Effects of endogenous and exogenous nitric oxide (NO) on SPARC and iNOS protein levels. Quiescent MC were treated as indicated for 24 h. Protein was separated on sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotted using iNOS- and SPARC-specific antibodies. Blots were developed using the enhanced chemiluminescence system (Amersham-Buchler) (A), and the intensities of the bands representing SPARC (43 kD) and iNOS protein (130 kD) were evaluated using densitometry (B). Data in Panel B are means ± SD obtained in three different experiments. $*P < 0.05$ *versus* control; $**P < 0.01$ *versus* control; $^{\#}P < 0.05$ *for IL-1β + L-NMMA versus IL-1β alone.*

to known sequences in commonly used databases was verified using Northern blotting. Reamplification, cloning, sequencing, and database searches of one excised band of 680 bp that showed a reverse transcription pattern comparable to iNOS resulted in the identification of the regulated transcript as SPARC cDNA.

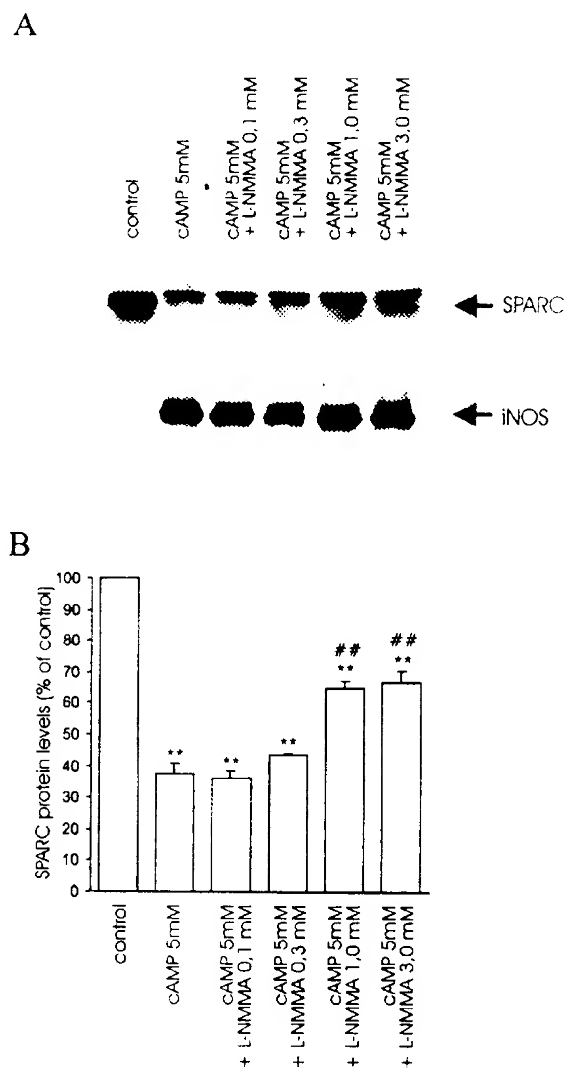

Figure 5. Modulation of SPARC protein expression by cAMP. MC were grown to subconfluence in 10-cm dishes, maintained serum-free for 24 h, and then stimulated with the membrane-permeable cAMP analogue N^6,O-2′dibutyryladenosine 3′,5′-phosphate (Bt_2cAMP) (5 mM) and the indicated concentrations of L-NMMA for an additional 24 h. Cellular protein was then subjected to Western blot using SPARC- and iNOS-specific antibodies (A). The results of the densitometric evaluation of three independent experiments are shown in Panel B. $**P < 0.01$ *versus* control; $^{\#\#}P < 0.01$ *versus cAMP alone.*

In the kidney, SPARC expression is transiently enhanced as exemplified during anti-Thy1.1-mediated glomerulonephritis (14), in a subtotal nephrectomy model (36), and during interstitial fibrosis as observed in a model of Heymann nephritis (37). In glomerular MC, SPARC acts as an autocrine or paracrine inhibitor of PDGF-mediated proliferation (14). In human dermal fibroblasts, binding of SPARC to the B-chain of PDGF is probably the mechanism responsible for inhibition of the PDGF responses (38). The characterization of SPARC as an anti-mitogenic factor suggests a role in resolution of interstitial fibrosis in passive Heymann nephritis or resolution of MC

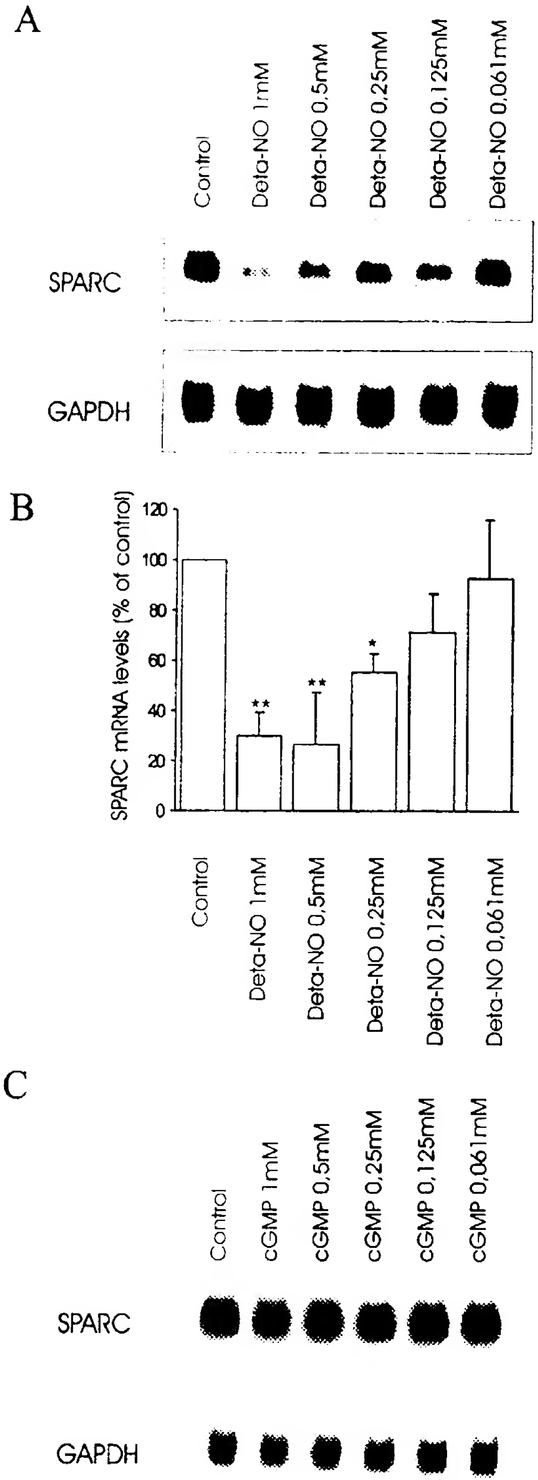

Figure 6. Effects of DETA-NO and cGMP on SPARC mRNA-levels. MC that were maintained serum free for 24 h were treated with the indicated concentrations of DETA-NO (A and B) or 8-bromo-cGMP (C) for an additional 24 h. MC were harvested and total mRNA was subjected to Northern blotting (A). Densitometric evaluation of the DETA-NO effect is shown in Panel B. $*P < 0.05$ *versus* control. $**P < 0.01$ *versus* control. Data (mean ± SD) represent three independent experiments.

proliferation in Thy1.1 glomerulonephritis. Because NO production is a crucial event in the course of Thy1.1 glomerulonephritis (1,3), this further strengthens the role of NO in mediating the balance between MC proliferation and apoptosis (39). Pichler *et al.* (14) also reported that SPARC protein expression in glomeruli is reduced at day 2 of Thy1.1 glomerulonephritis when maximal NO expression occurs (3). Moreover, the recovery of SPARC peaks at day 5 of Thy1.1 glomerulonephritis (14), a time point when iNOS expression has vanished (3).

Two different pathways have been described that independently lead to the induction of iNOS transcription in MC (7). In this study, we show that an enhanced formation of NO in response to cAMP or IL-1β produced a downregulation of SPARC mRNA and protein. The fact that the effects of both cAMP and IL-1β were reversed by L-NMMA suggests that NO is the mediator responsible for the observed downregulation of SPARC. In accordance with our previous report (10), we observed here that the expression of IL-1β-induced iNOS protein was downregulated by L-NMMA (Figure 4). However, in contrast to SPARC, which can be downregulated by NO alone, modulation of iNOS expression requires additional IL-1β-triggered signals.

Regulation of SPARC expression appears to be very complex and displays species- and cell-specific specificity, as well as developmental control features, and includes transcriptional and posttranscriptional mechanisms (reviewed in reference (13). Although the murine, bovine, and human SPARC genes and their 5′ flanking regions have been cloned and characterized, the transcriptional regulation of SPARC is not yet fully understood. NO has been shown to influence the action of redox-sensitive transcription factors (*e.g.*, NF-κB and AP-1) (reviewed in reference (29). However, corresponding transcription factor-binding sites in the SPARC promoter regions of different species, to our knowledge, have not yet been identified. Future studies are needed to elucidate the role of NO in transcriptional and posttranscriptional regulation of SPARC expression in mammals.

The *in vivo* relevance of our data has been tested in a rat model of endotoxemia. Administration of LPS produced a significant decrease in MAP after 4 h, and as described previously (35), LPS also induced iNOS expression in whole kidneys in a time-dependent manner (an effect that was accompanied by a decrease in the protein levels of SPARC) (Figure 7). Treatment of the rats with the selective and specific iNOS inhibitor L-NIL both attenuated the LPS-mediated hypotension and reversed the reduction of SPARC expression, suggesting that this effect is indeed mediated by endogenously produced NO. Based on our results in MC that are strengthened by the endotoxemia model, additional experiments are in progress to evaluate whether NO-mediated changes in SPARC expression are implicated in experimental forms of glomerulonephritis.

Acknowledgments

We thank Dr. J. Cunningham (Boston, MA), Dr. Ch. Rordorf (Novartis Pharma, Inc., Basel, Switzerland), and Dr. R. Timpl (Martinsried, Germany) for providing the mouse cDNA clone pMAC-

Table 1. Effects of LPS and L-NIL on MAP and HR in anesthetized rats[a]

Group	Baseline	30 min	1 h	2 h	4 h
LPS + saline					
MAP (mmHg)	122 ± 15	81 ± 5[b]	94 ± 8[b]	104 ± 8[c]	85 ± 1[c]
HR (beats/min)	381 ± 37	372 ± 27	388 ± 32	388 ± 53	429 ± 42
LPS + L-NIL					
MAP (mmHg)	126 ± 8	93 ± 4[b]	103 ± 13[c]	106 ± 1[c]	101 ± 8[c,d]
HR (beats/min)	377 ± 31	410 ± 27	398 ± 33	403 ± 70	413 ± 25

[a] Rats received an intravenous injection of either LPS alone or LPS plus the selective iNOS inhibitor L-NIL. MAP and HR was measured at baseline and then 30 min, and 1, 2, and 4 h after LPS administration. Data are represented as mean ± SD. LPS, lipopolysaccharide; L-NIL, L-N^6-1 (iminoethyl) lysine dihydrochloride; MAP, mean arterial blood pressure; HR, heart rate; iNOS, inducible nitric oxide synthase.
[b] $P < 0.01$ *versus* baseline value.
[c] $P < 0.05$ *versus* baseline value.
[d] $P < 0.05$ *versus* LPS treatment.

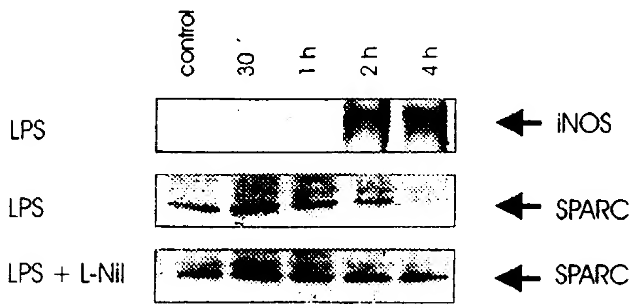

Figure 7. NO-mediated downregulation of SPARC protein expression in whole kidneys in a model of endotoxemia in rats. Wistar rats were treated with lipopolysaccharide (LPS) or LPS + L-NIL for the indicated time points. The animals were sacrificed and the kidneys were removed. Total protein extracts were subjected to Western blotting against iNOS and SPARC antibodies as indicated.

NOS, recombinant human IL-1β, and SPARC antibodies, respectively. Ute Schmidt's valuable technical assistance is gratefully acknowledged. The authors' work was supported by the Deutsche Forschungsgemeinschaft (SFB553) and by a grant from the Commission of the European Communities (Biomed 2, PL 950979). Prabal K. Chatterjee is supported by the Joint Research Board of St. Bartholomew's Hospital (Grant XMLA), and Christoph Thiemermann is a Senior Fellow of the British Heart Foundation (FS 96/018).

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ARTICLE

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The impact of osteonectin for differential diagnosis of osteogenic bone tumors: an immunohistochemical and in situ hybridization approach

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Abstract Thirty-three osteosarcomas at various grades of histologic differentiation, including chondroblastic, osteoblastic, and fibroblastic variants, were investigated immunohistochemically for evidence of osteonectin. Twenty-two cases of varying types of osteosarcoma were examined with in situ hybridization for mRNA expression of osteonectin. Immunohistochemically, osteonectin was present in all the osteosarcomas in this study. With in situ hybridization, 12 out of 22 osteosarcomas showed a positive signal. Two osteochondrosarcomas, seven chondrosarcomas, and one mesenchymal chondrosarcoma were also studied with regard to the localization of osteonectin, either immunohistochemically or by in situ hybridization. Immunohistochemically, osteonectin was present in all the chondroid lesions except for one osteochondroma. However, in situ hybridization of osteonectin mRNA was negative in all the chondroid lesions we studied. This study revealed that immunohistochemical localization of osteonectin is not useful in providing conclusive diagnosis of osteosarcoma. In situ hybridization of osteonectin mRNA might be useful in differentiating osteosarcoma from nonosteogenic bone tumors.

Key words Osteonectin · Osteosarcoma · In situ hybridization · Immunohistochemistry

Introduction

Osteonectin (ON) is a bone-specific, phosphorylated glycoprotein with a molecular weight of 32 kDa [1]. It accounts for approximately one-fourth of all noncollagenous bone matrix proteins, which in turn make up 10% of the total protein content of the bone matrix. The name "osteonectin" is derived from the affinity of this protein for the collagenous bony constituents of the matrix and for the mineral hydroxyapatite [2]. Osteonectin is produced by the osteoblast and has been demonstrated immunocytochemically in active, bone-matrix-producing cells (osteoblasts and young osteocytes) but not in dormant osteocytes or inactive superficial endosteal cells [3]. For this reason, osteonectin can be regarded as a marker in the differentiation of bone-forming cells, and it was therefore logical to test its marker characteristics on bone-forming tumor cells [4].

Diagnosis of osteosarcoma becomes difficult, however, if the tumor shows no matrix production, or if matrix production is scanty and hard to recognize. This problem arises in particular if the diagnosis has to be established on the basis of a small biopsy, as has recently been proposed in most of the limb salvage procedures. In such cases, demonstration of osteonectin by immunohistochemical or in situ hybridization methods would be important and helpful, in addition to purely morphologic criteria, for establishing the osteoblastic origin of a tumor.

We investigated the presence of osteonectin in tissue sections obtained from primary bone tumors, using immunohistochemistry and in situ hybridization to evaluate the usefulness of this protein as a diagnostic marker of osteogenic tumors.

Materials and methods

Thirty-three osteosarcomas in various stages of differentiation from 29 patients were studied by immunohistochemistry. The tumors were subtyped according to their main component as osteoblastic, chondroblastic, and fibroblastic. Twenty-two osteosarcomas from 20 patients were studied by in situ hybridization for mRNA expression of osteonectin. Seven chondrosarcomas, one mesenchymal chondrosarcoma, and two osteochondromas were also studied by immunohistochemistry and in situ hybridization for osteonectin mRNA.

The antibody for osteonectin was obtained from Hematologic Technologies Inc. (Pinewood Plaza, Essex, Vermont). It was a mouse monoclonal IgG and could recognize human and bovine bone osteonectin.

An osteonectin antisense oligo-DNA probe was synthesized. This was a 50-mer complementary to the human mRNA sequence that begins with nucleic acid 1232 [5]. It was 5' TGT GTT TAA GGC AGA GCC CGA CAG ATC CGT GTC CAC CCA TGT GCC AAT AA3'.

Immunohistochemical method

Immunolocalization was performed using a streptavidin-biotin immunoperoxidase method, as previously described (DAKO LSAB kit, Carpinteria, California). Briefly, 6-μm paraffin sections were fixed to silanized slides (DAKO, Carpinteria, California) and dried. After deparaffinization and rehydration, the tissue sections were incubated for 5 min with a 3% hydrogen peroxide and blocking reagent. The sections were exposed to the primary antibodies (1:200 working dilution with DAKO antibody diluent) for 30 min at 37°C. After washing with Tris-buffered saline (DAKO), biotinylated link antibody was applied for 15 min, followed by streptavidin peroxidase for an additional 10 min. Color development was performed with substrate-chromogen (3-amino-9-ethylcarbazole) solution for 10 min.

In situ hybridization

A ^{35}S-ATP-labeled single-stranded antisense for cDNA was prepared with terminal deoxynucleotidyl transferase using a NEP-100 labelling kit (Du Pont, Boston, MA). The ^{35}S-labeled probe was used for hybridization at a concentration of 5.6 kcpm/ml. Treatment of the slides and hybridization conditions were as described elsewhere [6], with our own modification. After hybridization, the sections hybridized with osteonectin were treated with 10 mg/ml RNase in 5 M NaCl, 1 M Tris, pH 8.0, 0.5 M EDTA at 37°C for 30 min. Sections were washed twice in 2×SSC, once in 1×SSC, and once in 0.5×SSC. They were then dehydrated in graduated ethanol and dipped in NTB-2 emulsion (Eastman Kodak, Rochester, New York) diluted 1:1 with pure water. The dipped slides were placed in a well-ventilated area for 1 h to dry at room temperature, and were exposed at 4° C in dessicated slide boxes. The exposed slides were developed in a D-19 developer for 5 min at room temperature, fixed in a fixative for 5 min, and washed with water for 20 min. They were counter-stained with hematoxylin and eosin.

Results

Immunohistochemical study of osteonectin

We evaluated the expression of osteonectin in paraffin-embedded sections obtained from surgical specimens of various bone tumors (Table 1) using immunohistochemistry. All the osteosarcomas, of various histologic subtypes, reacted positively with the antibody against human and bovine osteonectin. The intensity of reaction differed, but there were no differences between the tumor subtypes (Table 1). The classical osteoblastic osteosarcomas composed of tumor cells and neoplastic osteoid showed grade 1+ to grade 3+ staining intensity in the osteoblastic cytoplasm (Fig. 1). The chondroblastic osteosarcomas with abundant cartilage formation showed positive marking of neoplastic chondrocyte cytoplasm at variable staining intensities (Fig. 2). The fibroblastic osteosarcomas revealed variable staining intensities in the tumor cells (Fig. 3).

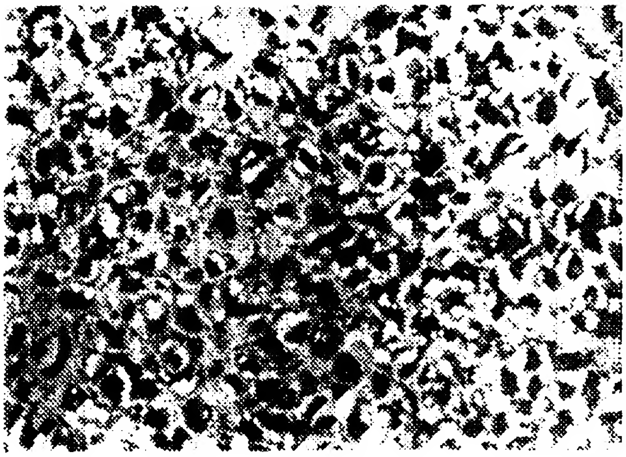
Table 1 Intensity of immunohistochemical reaction of osteonectin antibodies in osteosarcomas and nonosteogenic bone tumors

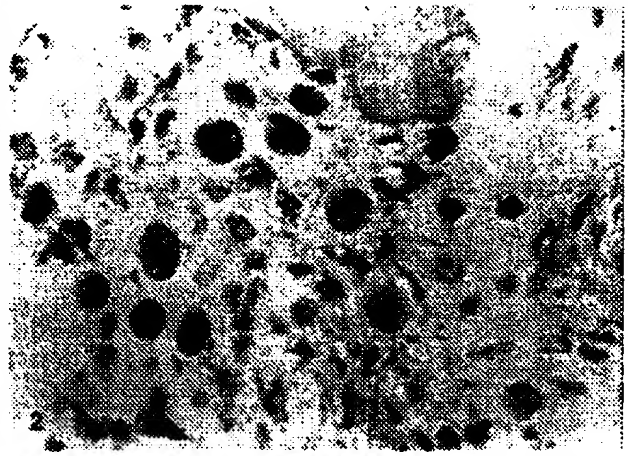
Diagnosis	No. of cases	Reaction intensity
Osteosarcoma		
Osteoblastic	4/14	+
	5/14	++
	5/14	+++
Chondroblastic	6/10	++
	4/10	+++
Fibroblastic	2/8	+
	6/8	+++
Nonosteogenic tumors		
Chondrosarcoma	1/7	+
	3/7	++
	3/7	+++
Mesenchymal chondrosarcoma	1/1	++
Osteochondroma	1/2	–
	1/2	+

Immunohistochemically, scattered giant cells were negative for osteonectin antibody. Seven grade II chondrosarcomas were studied. One showed focal grade 1+ staining in the cytoplasm of the neoplastic chondrocytes, three revealed grade 2+ staining intensity, and the remaining three showed grade 3+ intensity in the cytoplasm of the neoplastic chondrocytes. Of the two osteochondromas, one showed focal positive reaction in the cytoplasm of chondrocytes and the other was negative. One mesenchymal chondrosarcoma showed grade 2+ staining intensity in neoplastic chondrocytes. The hemangiopericytomatous area of mesenchymal chondrosarcoma showed grade 2+ staining intensity.

In situ hybridization of osteonectin mRNA expression

Of the 14 cases of osteoblastic osteosarcomas, eight were used for in situ hybridization (Table 2). Four were totally negative for the osteonectin signal, one was grade 1+, one was grade 2+, and two cases were grade 3+ for osteonectin mRNA expression in the neoplastic osteo-





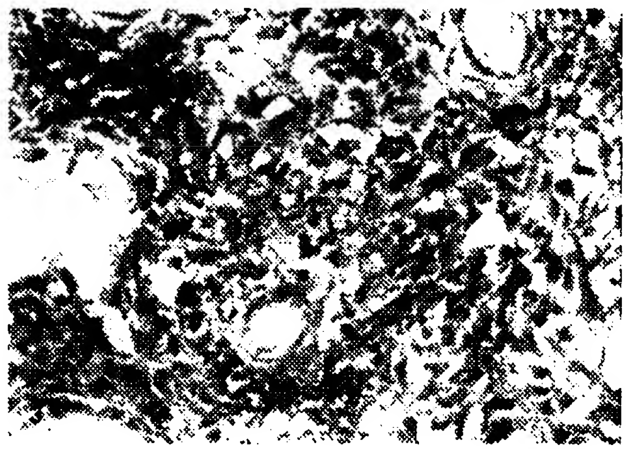


Fig. 1 Osteoblastic osteosarcoma showing grade 3 positivity for osteonectin in the cytoplasm of the neoplastic tumor cells. (×200)

Fig. 2 Chondroblastic osteosarcoma showing grade 3 positivity for osteonectin in the cytoplasm of the neoplastic chondrocyte. (×200)

Fig. 3 In fibroblastic osteosarcoma, there is diffuse grade 3+ osteonectin immunostaining in the tumor cell cytoplasm. (×200)

Table 2 In situ hybridization results of various osteosarcomas and nonosteogenic bone tumors

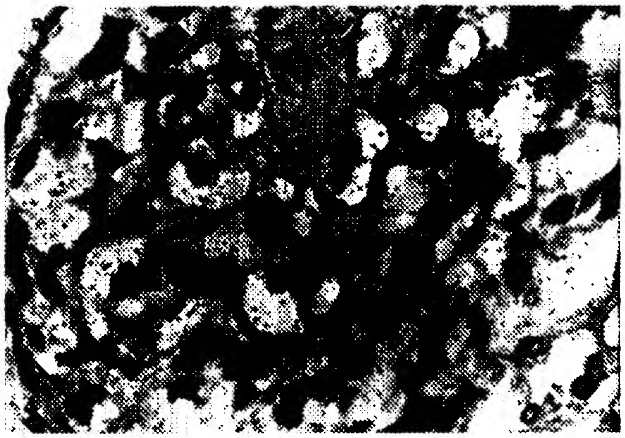
Histologic type	No. of cases	Reaction intensity
Osteosarcoma		
Osteoblastic	4/8	–
	1/8	+
	1/8	++
	2/8	+++
Chondroblastic	6/7	–
	1/7	+
Fibroblastic	1/7	+
	5/7	++
	1/7	+++
Nonosteogenic tumors		
Chondrosarcoma	7/7	
Mesenchymal chondrosarcoma	1/1	+[a]
Osteochondroma	2/2	–

[a] Only spindle cell areas focally positive

blasts (Fig. 4). Among the ten cases of chondroblastic osteosarcoma, seven used for in situ hybridization. Six were negative for the osteonectin signal. One case showed grade 3+ reaction for osteonectin mRNA expression in the neoplastic chondroid area. Seven patients with fibroblastic osteosarcoma were studied by in situ hybridization. One patient was grade 1+, five patients were grade 2+, and one tumor showed a grade 3+ signal for osteonectin mRNA expression in the cytoplasm of neoplastic spindle cells (Fig. 5). Two osteochondromas and seven grade II chondrosarcomas were negative for the osteonectin signal. One mesenchymal chondrosarcoma showed a negative signal over the cartilage island and a focal grade 1+ signal in the hemangiopericytomatous area

Discussion

Osteonectin is a noncollagenous protein of bone which is believed to be specific for bone since its concentration in bone is 500–1000 times higher than in other connective



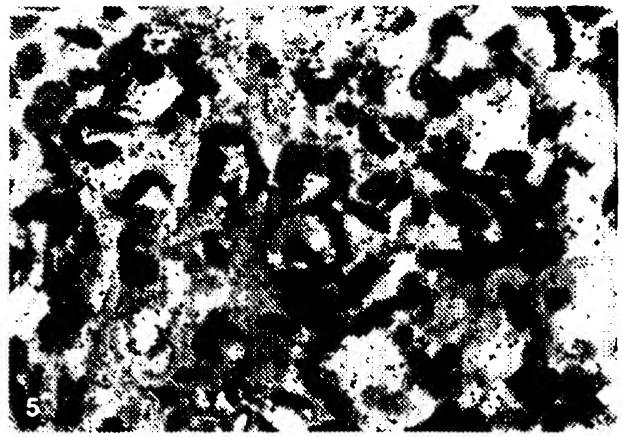


Fig. 4 Diffuse grade 3+ staining of the neoplastic osteoblasts in osteoblastic osteosarcoma (in situ hybridization for osteonectin, ×400)

Fig. 5 Diffuse grade 3+ reactions of the fibroblastic osteosarcoma cells (in situ hybridization for osteonectin, ×400)

tissues [7]. The functional importance of osteonectin in bone is unknown. It has been found to have a strong affinity for calcium hydroxyapatite molecules as well as for collagen fibrils, thus linking the two structures [7]. Its probable function is to maintain bone structure by contributing to the stabilization of the hydroxyapatite molecule and to its organization in the fibrillar collagen matrix [8]. Antibodies against osteonectin have been used successfully for the characterization of bovine and human bone cell cultures [9, 10], in a study of dental tissue [11], and in reactive bone lesions [3].

Osteoblasts differ from other connective tissue cells in their ability to form bone matrix. Immunohistochemical studies on the localization of osteonectin in fetal bovine [1, 12] and human bone [3, 12] showed that osteonectin was localized to the cytoplasm of osteoblasts and osteoprogenitor cells, as well as in young osteocytes and newly synthesized osteoid in the bone matrix. It has therefore been suggested to be a reliable differentiation marker of osteogenic bone cells [3].

In this study, osteonectin was shown immunohistochemically to be expressed in all the osteosarcomas, whatever their histologic subtype. These results are similar to those of previous studies [2, 7, 13, 14]. However, seven chondrosarcomas we studied immunohistochemically did react with the osteonectin antibody, in the cytoplasm of chondrocytes. Of two cases of osteochondromas one showed a positive reaction for the osteonectin antibody in the cytoplasm of chondrocytes. Another osteochondroma revealed a positive reaction in the osteocytes' cytoplasm. One mesenchymal chondrosarcoma showed a positive reaction in both the chondrocytes and in hemangiopericytomatous areas. Bosse et al. [13] demonstrated positive reaction in the chondrosarcoma they studied. Immunohistochemically, our results in chondrosarcomas and osteochondromas are not in agreement with those of other workers [3, 4, 14]. Chondrocytes in the metaphyseal growth plate also stained positively using antibodies against osteonectin. In cartilage, osteonectin was found in the mineralizing zone but at a relatively low level in resting, proliferating, and early hypertrophic zones, where osteonectin was mainly cell-associated [15]. Pacifici et al. [15] stated that osteonectin was demonstrated to be synthesized by cells in both nonmineralizing and mineralizing zones, indicating that osteonectin was produced by chondrocytes in each zone, but preferentially accumulated in the mineralizing zone. Bianco et al. [12], Metsaranta et al. [16], and Mundlos et al. [17] reported osteonectin signal in chondrocytes. Serra et al. [14] pointed out that the reported positivity found by other investigators in chondrosarcoma, Ewing's sarcoma, and fibrosarcoma might be attributed to differences in antibody concentrations and in the immunohistochemical methods used. We used a 1:200 dilution of the primary antibody and performed immunohistochemistry using the streptavidin-biotin immunoperoxidase method. We used a much higher concentration that others (1:400 [13] and 1:1200 [14]). Thus immunohistochemistry for osteonectin is not a specific diagnostic tool for making the diagnosis of osteosarcoma.

According to our study, of the cases of osteoblastic osteosarcoma, half were positive for the osteonectin mRNA signal with in situ hybridization. Only one chondroblastic osteosarcoma was positive out of seven, and all seven fibroblastic osteosarcomas were positive for the osteonectin mRNA signal. Two osteochondromas and seven grade II chondrosarcomas were negative for the osteonectin signal. In the case of mesenchymal chondrosarcoma, the cartilage area was negative but the hemangiopericytomatous area was positive for the mRNA signal. These findings suggest that osteonectin mRNA expression does not help to make the diagnosis in osteosarcoma with abundant chondroid tissue. However, the osteonectin mRNA study is helpful in making the diagno-

sis of fibroblastic osteosarcoma in conjunction with histologic findings.

In situ hybridization of developing human bones [16] and mouse condyles [18] revealed that osteoblasts, cells in the periosteum, and hypertrophic chondrocytes expressed osteonectin at high levels. In this study, we used an oligonucleotide probe for in situ hybridization on the decalcified bone tissue.

The differences in osteonectin expression shown by immunohistochemical study and in situ hybridization may be due to tissue processing. Immunohistochemical demonstration of osteonectin expression reveals osteonectin protein itself. This may be more stable than the mRNA level. During decalcification and fixation, the protein lies inside the cells, but the mRNA for osteonectin may be destroyed by strong acid treatment for decalcification.

This study has shown that immunohistochemical localization of osteonectin is not useful in confirming a diagnosis of osteosarcoma. However, osteonectin mRNA expression as determined through in situ hybridization may be useful in making a differential diagnosis of osteosarcoma.

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Immunological Screening of SPARC/Osteonectin in Nonmineralized Tissues

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Abstract

SPARC/Osteonectin is a major bone-related protein that is also present in nonmineralized tissues and in platelets. As compared to bone SPARC/Osteonectin, SPARC/Osteonectin from platelets presents a slightly lower electrophoretic mobility in SDS-PAGE and a 100-fold decreased affinity for a unique monoclonal antibody, Mab2 (Malaval et al. 1991). To check the tissular diversity of SPARC/Osteonectin, protein extracts from bovine bone, nonmineralized tissues, and platelets were screened by immunoblotting and immunoradiometric assay, with Mab2 and three other monoclonal antibodies recognizing distinct epitopes. The SPARC/Osteonectin secreted by a human osteosarcoma cell line (MG63) was also tested. In all the nonmineralized tissues tested (gut, bone marrow, tendon, mesentery, artera, lens, skin, liver, and cornea), SPARC/Osteonectin presents the same immunoreactivity and electrophoretic mobility as in bone. The heavier molecular weight and Mab2-negative form present in platelets seems to be unique to this cell type. Osteosarcoma cell extracts and conditioned media give the same results as bone extracts, indicating that the low molecular weight and Mab2-positive form of SPARC/Osteonectin present in most tissues does not result from proteolytic cleavage in the matrix, but is secreted as such. Bone and platelet SPARC/Osteonectin present different patterns of sensitivity to glycosidases, suggestive of a difference in *N*-glycosylation. However, these treatments do not affect the decreased affinity of Mab2 for platelet SPARC/Osteonectin, which is not likely to be related to difference in *N*-glycosylation.

Key Words: SPARC—Osteonectin—Monoclonal antibodies—Bone—Platelets.

Introduction

Osteonectin (Mr = 30.000) is one of the major noncollagenous proteins of bone tissue. This phosphoglycoprotein presents a high affinity for hydroxyapatite and type I collagen (Termine et al. 1981a; 1981b). Although it was originally believed to be specific of bone, further works (Wasi et al. 1984; Tung et al. 1985; Young et al. 1986; Malaval et al. 1987) have shown its presence and synthesis in many nonmineralized tissues. Osteonectin was found to be identical to a 43-kD protein secreted by bovine aortic endothelial cells when subjected to cellular stress (Sage et al. 1984), to BM-40, extracted from a mouse Englebreth-Holm-Swarm tumor (Dziadek et al. 1986) and to secreted protein, acidic and rich in cystein (SPARC), found in mouse embryo parietal endoderm (Mason et al. 1986) and human placenta (Swaroop et al. 1988). SPARC/Osteonectin is also secreted by platelets (Stenner et al. 1986). Study of α–granule-deficient platelets (Clezardin et al. 1991) as well as PCR amplification of mRNA from megakaryocytes (Villareal et al. 1991a) have recently established that osteonectin is present in this cell type due to synthesis, and not by uptake from extracellular medium. The compound name SPARC/Osteonectin takes into account the widespread distribution of this protein in tissues and organs, as well as its specific accumulation in bone. This highly conserved protein is encoded by a single gene in all species studied (McVey et al. 1988; Findlay et al. 1988; Swaroop et al. 1988; Villareal et al. 1989). We have recently developed monoclonal antibodies (Mabs) raised against bovine bone SPARC/Osteonectin (Malaval et al. 1991). One of them, Mab2, presents a 100-fold lower affinity for SPARC/Osteonectin from platelets. Platelet SPARC/Osteonectin also exhibits a slightly lower electrophoretic mobility than SPARC/Osteonectin from bone (Kelm & Mann 1990; Malaval et al. 1991). Thus, despite the RNA transcript identity of SPARC/Osteonectin in human bone and nonmineralized tissues (Villareal et al. 1989), and the identity of mRNA size in human bone and platelets (Villareal et al. 1991a), structural differences exist between secreted proteins. To assess the extent of this variability, we have screened SPARC/Osteonectin in platelets, nonmineralized tissues and in an in vitro bone cell model, with four Mabs recognizing four distinct epitopes, using immunoblotting and immunoradiometric assay (IRMA). To further describe the differences that exist between the two forms of SPARC/Osteonectin, we have studied the influence of the carbohydrates moieties on the immunoreactivity of the Mabs and tested the ability of platelet SPARC/Osteonectin to bind to hydroxyapatite.

Materials and Methods

Extraction of bone and nonmineralized tissues

Bovine long bone was dissected from preterm fetuses. Human bone was a femur from a 20-year-old man who died in an accident. Material secreted by human platelets, obtained after thrombin stimulation of concentrated pools as previously described (Clezardin et al. 1988), was a gift of Dr. P. Clezardin, Inserm U331, Lyon, France. Bovine bone SPARC/Osteonectin was extracted in denaturing conditions, according to Termine et al. (1981a), as described previously (Malaval et al. 1987). Briefly, fresh bone was powdered in liquid nitrogen, rinsed in distilled

water and then acetone, and vacuum dried. The powder was extracted in 4 M guanidium chloride, 50 mM Tris/HCl, pH 7.4, containing protease inhibitors (β-aminohexanoic acid 0.1 M, benzamidinium chloride 5 mM, phenylmethansulfonyl fluoridate 0.5 mM) for 24 hours and then for three days in the same medium with 0.5 M EDTA. All chemicals were purchased from Merck (Darmstadt, Germany). After centrifugation, the supernatant of the second extract, containing bone proteins, was concentrated by ultrafiltration, desalted, and lyophilized. Nonmineralized tissues were dissected from preterm calf fetuses. The extraction was performed in denaturing conditions in a guanidium chloride medium containing protease inhibitors (see above). The extracts were concentrated, desalted, and lyophilized as described for bone extracts.

Purification of bone and platelet SPARC/Osteonectin

Purification of bovine bone SPARC/Osteonectin was achieved by submitting the extracts to repeated ion-exchange chromatography on DEAE sephacel equilibrated in 7 M urea, 50 mM Tris, pH 7.4, eluted with a 0.1–0.26 M NaCl gradient, and gel filtration chromatography on Sepharose CL6B in 4 M guanidium chloride, 50 mM Tris, pH 7.4. All chromatography media were purchased by Pharmacia (Uppsala, Sweden). The purity of isolated proteins was checked by Tricine sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) performed according to Schägger and Jagow (1987) using 7.5/3 separating gels (the first figure gives the percentage of total acrylamide in the mixture and the second figure gives the percentage of bisacrylamide versus total acrylamide) and 4/3 stacking gels. Gels were run in a miniprotein cell (Bio-Rad, Richmond, CA, USA) under 100 V at room temperature using a Tris 0.2 M, pH 8.9, as an anode buffer and a Tris 0.1 M, Tricine 0.1 M, pH 8.25, as a cathode buffer. All chemicals were obtained from Merck, except for Tricine which was purchased from Serva (Heidelberg, Germany).

Human platelet SPARC/Osteonectin was purified as previously described (Clezardin et al. 1988). Briefly, human platelet extracts were dialyzed against a Tris/urea buffer and submitted to the same steps of chromatography as described above. As SPARC/Osteonectin concentration in the extracts was variable and sometimes low, fractions were screened with the polyclonal antiserum-based radioimmunoassay (RIA) previously described (Malaval et al. 1987). Purified bovine bone sialoprotein was a gift of Dr. C. Chenu, Inserm U234, Lyon, France.

Isolation of bovine platelets

Bovine blood (6 vol) was drawn into acid/citrate/dextrose anticoagulant (1 vol). The blood was centrifuged for 20 min at 160 *g* to obtain platelet-rich plasma. Platelets were pelleted by centrifugation at 1200 *g* for 15 min, and the pellet was washed three times in Tyrode's buffer (140 mM NaCl, 3 mM KCl, 12 mM $NaHCO_3$, 4 mM NaH_2PO_4, 1 mM $MgCl_2$, 5.5 mM glucose, pH 7.35) containing 0.35% BSA, 5 mM Hepes, 10 μl/ml apyrase and 50 U/ml heparin. After centrifugation, the pellet was washed in Tyrode's buffer without heparin and platelets were finally resuspended in Tyrode's buffer containing 2 μl/ml of apyrase.

Monoclonal antibodies

The production and characterization of a library of Mabs against SPARC/Osteonectin have been previously described (Malaval et al. 1991). Briefly, male Balb/C mice were immunized with degradation products of bovine bone SPARC/Osteonectin. Twelve of the hybridomas produced by the fusions were selected and grown as ascites. The Mabs were purified from the ascites on a Protein A-Sepharose column (Pharmacia, Uppsala, Sweden). Four distinct epitopes, or groups of epitopes, on SPARC/Osteonectin were recognized. Raising, producing, and purifying these antibodies were performed in Cis-Bioindustries (Bagnols-sur-Cèze, France). Titration of Mabs was performed by RIA, as described in this section.

Cell cultures

MG 63 human osteosarcoma cell line was purchased from Flow Laboratories (Puteaux, France). Secretion of osteocalcin and SPARC/Osteonectin, as well as stimulation of osteocalcin secretion by 1,25-dihydroxyvitamin D3, were previously assessed on these cells (Glibert et al. 1989). Cells were grown to confluency in 250-ml flasks (Falcon, Oxnard, CA, USA) in RPMI medium containing 10% fetal calf serum (FCS), 1% glutamine, and 1% streptomycin-penicillin. Cells were then trypsined, rinsed in 10 ml RPMI-10% FCS and transferred to 250-ml flasks. These cells were grown in a FCS-free RPMI medium for four days. On the fourth day, the culture medium was removed, aliquoted, and frozen. Cell lysis was performed by sonicating suspended cells in PBS buffer. All chemicals were purchased from Gibco (Paisley, Scotland).

Iodination procedure

Bone SPARC/Osteonectin, platelet SPARC/Osteonectin, bovine bone sialoprotein and Mabs were iodinated with the chloramin T method. Five micrograms of purified SPARC/Osteonectin were incubated in PBS 0.1 M with 0.5 mCi of ^{125}I in 0.4 mg/ml of chloramin T. The reaction was stopped by adding sodium metabisulfite (0.5 mg/ml in PBS 0.1 M). Iodinated proteins were separated from free iodine by gel filtration on sephadex G 25 (PD 10 column, Pharmacia, Uppsala, Sweden). Mabs were iodinated by the same method using 0.1 mCi of ^{125}I for 10 μg of purified Mab (Mab2: 4 μCi/μg; Mab3: 28 μCi/μg; Mab6: 12 μCi/μg; Mab12: 17 μCi/μg).

Immunoassay for SPARC/Osteonectin

The amounts of SPARC/Osteonectin in tissue extracts were assessed with two immunoassays. A competitive liquid phase radioimmunoassay was previously developed with a rabbit polyclonal antiserum raised against bovine bone SPARC/Osteonectin (Malaval et al. 1987). This assay, performed in equilibrium conditions, gives a typical linear range from 10 ng/ml to 1000 ng/ml of SPARC/Osteonectin.

A sandwich immunoradiometric assay (IRMA) using Mabs directed to bovine bone SPARC/Osteonectin and cross-reacting with human bone SPARC/Osteonectin has been recently developed (Malaval et al. 1991). Solid phase helices coated with Mab3 are first incubated in assay buffer (50 mM Tris/HCl, pH 8, 20 mM $CaCl_2$, 0.03% sodium azide) containing 1% BSA with either bovine bone SPARC/Osteonectin standard or samples. After three rinses in distilled water, the helices are further incubated with a iodinated antibody directed to another epitope (tracers). Radioactivity bound to the helices after three rinses in distilled water is assessed on a gamma counter (Riastar, Packard Instruments Company, Downers Grove, IL, USA). Each sample is assessed with two tracers. The first one, ^{125}I-Mab12, binds equally to bone and platelet SPARC/Osteonectin. The second one ^{125}I-Mab2 presents a 100-fold lower affinity for platelet

SPARC/Osteonectin than for bone SPARC/Osteonectin (Malaval et al. 1991).

Immunoblotting

Aliquots of lyophilized tissue extracts or cell culture conditioned medium were dissolved in electrophoresis buffer and subjected to Tricine SDS-PAGE on a 7.5/3 polyacrylamide gel (Shägger & Von Jagow 1987). Electroblotting of these samples onto nylon-nitrocellulose membrane (Hybond-C-extra, Amersham, UK) was performed according to Towbin et al. (1979), with modifications. Nitrocellulose strips were then saturated for 1 h at room temperature in a Tris/calcium buffer (50 mM Tris/HCl, pH 8, 20 mM $CaCl_2$, 0.03% sodium azide) containing 5% bovine serum albumin (BSA). The strips were incubated with labelled Mabs in buffer containing 1% BSA (activity: 500,000 cpm/strip) for 12 hours at room temperature then rinsed (6 × 10 minutes) in Tris buffer saline (20 mM Tris, pH 7.4, 0.3 M NaCl) containing 0.05% Tween-20. Autoradiography plates were exposed to the strips for 24 h at −70°C before development.

Hydroxyapatite binding

Plastic tubes received 1 mg of hydroxyapatite (type IV, Sigma, St. Louis, MO, USA) and 0.4 ml of Tris buffer (50 mM Tris/HCl; 0.15 M NaCl; pH 8) containing 100,000 cpm of iodinated SPARC/Osteonectin (10 μCi/μg). In some assays, BSA (0.2 to 2 mg/ml) was added as a competitor. The tubes were incubated for 90 min at room temperature with constant stirring, centrifuged, and decanted. The activity of supernatants and pellets was measured on a gamma counter (Riastar, Packard Instruments Co., Downers Grove, IL, USA).

Enzymatic deglycosylation

SPARC/Osteonectin purified from bovine bone and human platelets was treated with endoglycosidase F (Endo F) and glycopeptidase F (PNG F), two endoglycosidases active on *N*-linked oligosaccharides of glycopeptides with different specificities; PNG F cleaves all the *N*-linked oligosaccharides except those attached to an *N* or *C*-terminal asparagine, while Endo F cleaves only high-mannose structures and biantennary hybrid and complex structures at the concentration used. These enzymes were purchased from Boehringer Mannheim Biochemica (Mannheim, Germany). Each tube received 1.5 ml of Endo F assay buffer (0.1 M sodium acetate, pH 5.2, 20 mM EDTA, 1% Triton X-100) or PNG F assay buffer (50 mM potassium phosphate, pH 7, 20 mM EDTA, 1% Triton X-100, 0.2% SDS); 1.5 μl of Endo F (0.05 U in 20 mM potassium phosphate, pH 7.2, 50 mM EDTA), or 5 μl of PNG F (1 U in 20 mM potassium phosphate, pH 7.2, 50 mM EDTA); 100,000 cpm of labeled bone (16 μCi/μg) or platelet (10 μCi/μg) SPARC/Osteonectin and was incubated for 12 hours at 38°C. Control tubes received no enzymes, or were incubated with 1% SDS instead of Triton X-100. Positive controls of deglycosylation were performed using iodinated bovine bone sialoprotein. After endoglycosidases treatment, an aliquot of each sample was subjected to Tricine SDS-PAGE as described above. Gels were then dried and the tracers revealed by autoradiography. Deglycosylation was assessed by the decrease of the apparent molecular weight after treatment with endoglycosidases.

The influence of deglycosylation on immunoreactivity was assessed by RIA. Fifty microliters of each sample was added to 50 μl of assay buffer (50 mM Tris/HCl, pH 8, 20 mM $CaCl_2$, 150 mM NaCl, 1% BSA) and 100 μl of the Mab tested at titer dilutions (Mab 2: 1 μg/ml; Mab 3: 0.5 μg/ml; Mab 6: 1 μg/ml; Mab 12: 5 μg/ml). Incubations were performed for 24 hours at 4°C. Mabs were then precipitated by adding 50 μl of sheep anti-mouse serum and 50 μl of a 1/40 dilution of bovine serum in assay buffer. After 20 minutes at room temperature, 1 ml of 4% Polyethyleneglycol 6000 in assay buffer was added in each tube. The tubes were then mixed, spun, and decanted, and the antibody-bound radioactivity present in the pellet was counted.

Results

Immunoblotting on tissue extracts

A set of 12 Mabs directed against bovine bone SPARC/Osteonectin has been raised which recognizes four epitopes or groups of epitopes named A to D (Malaval et al. 1991). Mab 12, recognizing epitope A, was used in the IRMAs but not in the immunoblots because its immunoreactivity is decreased by the reduction of the molecule (not shown). Autoradiography of strips incubated with ^{125}I-Mab2 recognizing epitope D, ^{125}I-Mab3 recognizing epitope C, and ^{125}I-Mab6 recognizing epitope B reveals a single immunoreactive band at Mr 41 kD in both bovine bone and nonmineralized tissues extracts. As shown previously with human platelets, no immunoreactive band was observed with ^{125}I-Mab2 in bovine platelets (**Fig. 1**). In addition, the immunoreactive band present in bovine platelets incubated with ^{125}I-Mab3 shows a slightly lower electrophoretic mobility than the immunoreactive band present in bovine bone extracts. In some cases, a high molecular weight immunoreactive band is present in tissue extracts. This band was found unstable and dependent on the batch of extract used. An immunoreactive band for ^{125}I Mab2 is also present in the mesentery extract at 35 kD. This band is a major degradation product, often observed in bovine bone SPARC/Osteonectin preparations (Malaval et al. 1991).

Immunoassays on tissue extracts

SPARC/Osteonectin was present in all the bovine nonmineralized tissues assayed, but the amounts determined with the two IRMA or with the RIA were 100- to 1000-fold lower than in bone tissue, i.e., 10 to 100 ng/100 μg of total nonmineralized tissue proteins versus about 8 μg/100 μg of total bone proteins (**Table I**). The amounts of SPARC/Osteonectin given by the IRMA with the two tracers used (^{125}I Mab2 and ^{125}I Mab 12) were significantly different only in bovine platelet extracts where SPARC/Osteonectin could not be detected with the Mab3/Mab2 IRMA (Table I).

Immunoblotting on conditioned medium

In this experiment, samples containing the same amounts of SPARC/Osteonectin (10 ng as assessed by RIA) were run on SDS-PAGE and blotted. Human bone extract and human platelet extract were used as controls of the same species. Autoradiography of strips (**Fig. 2**), incubated with ^{125}I Mab 2, Mab 3, and Mab 6 revealed a single immunoreactive band at Mr 41 kD in the cell medium sample. This band co-migrates with the immunoreactive band observed in human bone extract incubated with Mab3 but not with the immunoreactive band observed at 47 kD in human platelets extract incubated with Mab3. A major immunoreactive band co-migrating with SPARC/Osteonectin labeled in the human bone extract was also observed with the three Mabs in the lysate of MG 63 cells. In addition, a minor immunoreactive band, presenting a lower electrophoretic mobility (54 kD)

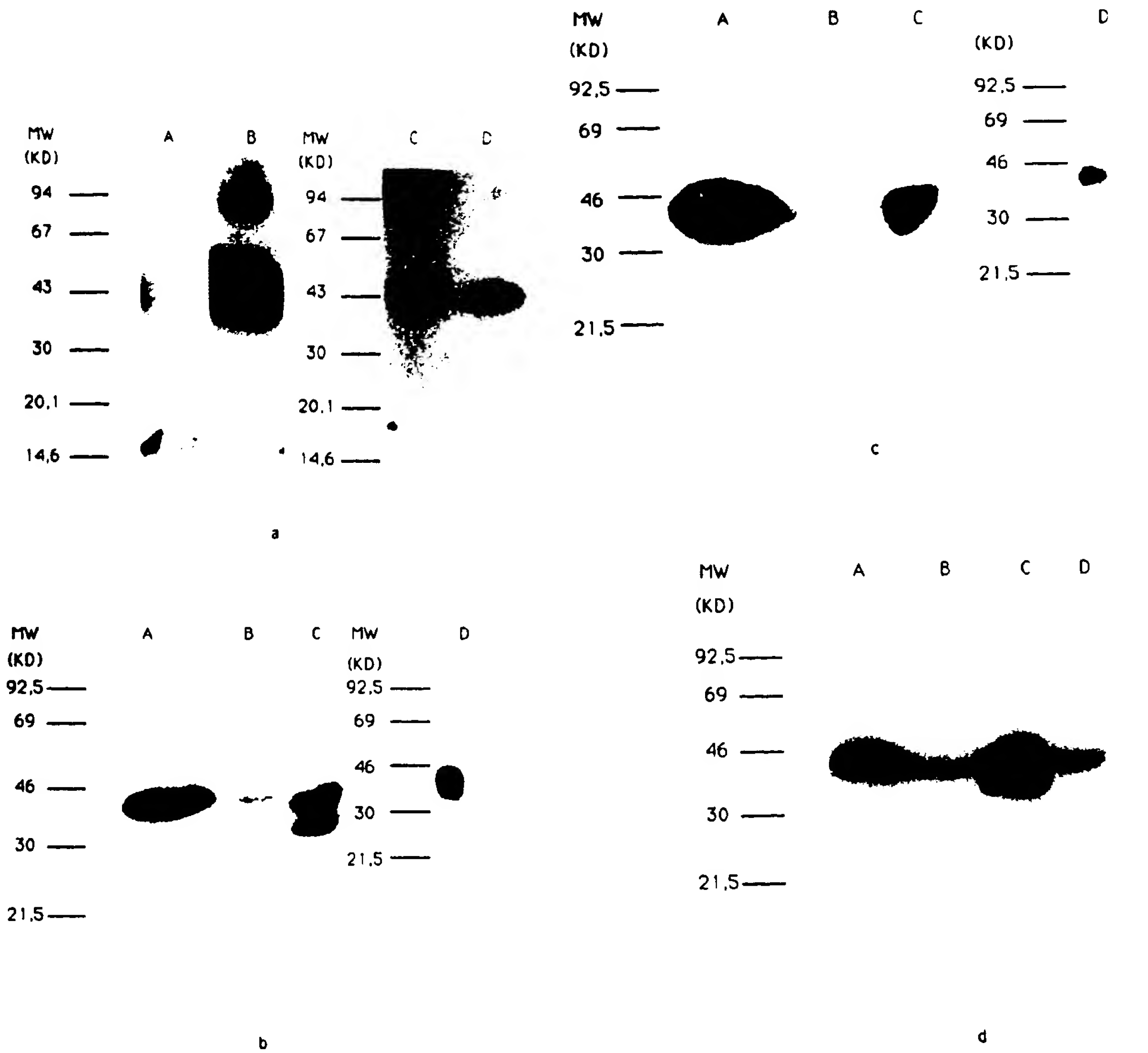

Fig. 1. Autoradiography of electroblots of protein extracts labeled with anti-osteonectin Mabs. (a) Comparison of immunoreactivity and electrophoretic mobility between bovine bone and platelet osteonectin incubated with ^{125}I-Mab2 (lanes A and B) and ^{125}I-Mab3 (lanes C and D). Lanes A and C: Bovine platelets. Lanes B and D: Bovine bone extract. (b)–(d) Labeling of bovine bone and nonmineralized tissue extracts with ^{125}I-Mab2 (b), ^{125}I-Mab3 (c) and ^{125}I-Mab6 (d). Lane A: Bone. Lane B: Tendon. Lane C: Mesentery. Lane D: Artera. Gels were prepared and run according to the method of Schägger and Von Jagow (1987) under reducing conditions. Blots were performed according to Towbin et al. (1979) (see Methods). MW, Molecular weight standards.

than platelet SPARC/Osteonectin, was labeled in the cell lysate by Mab3 (Fig. 2).

Enzymatic deglycosylation

Deglycosylation of SPARC/Osteonectin was assessed by the difference of electrophoretic mobility in SDS-PAGE between control and deglycosylated SPARC/Osteonectin (**Fig. 3**). The loss of apparent molecular weight of bone sialoprotein used as a positive control assures that the enzymatic activity was intact during the experiment (Chenu & Delmas 1992). Bovine bone SPARC/Osteonectin was deglycosylated by both endoglycosidases (Fig. 3, lanes B and D), endoglycosidase F, and glycopeptidase F while human platelet SPARC/Osteonectin was not deglycosylated by endoglycosidase F (Fig. 3, lane G). The immunoreactivity of bone and platelet SPARC/Osteonectin assayed with the four Mabs (Mab2, 3, 6, 12) is given in **Table II.** The immunoreactivity of treated samples was often decreased, as compared to controls, but was not consistent when compared to negative controls (i.e., glycosidases + 1% SDS). This may reflect a partial degradation of the tracer during incubation. The difference in immunoreactivity of bovine bone SPARC/Osteonectin and human platelet SPARC/Osteonectin assayed with Mab2 was not affected by the treatment with endoglycosidases. In SDS-PAGE, however, platelet SPARC/Osteonectin deglycosylated with glycopeptidase F co-migrates with bone SPARC/Osteonectin (Fig. 3, lanes I and A).

Hydroxyapatite binding

The ability of platelet and bone SPARC/Osteonectin to bind to hydroxyapatite was assessed by an in vitro method. No major

Table 1. Immunoradiometric assays with two immunological configurations and RIA for bovine fetal nonmineralized tissues and bone as well as bovine platelets*

Materials	RIA	IRMA MAB3/MAB2	IRMA MAB3/MAB12
Fetal bovine tissues			
Gut	67 ± 3	159 ± 6	188 ± 4
Bone marrow	53 ± 8	62 ± 3	42 ± 7.4
Tendon	38 ± 1	28 ± 3	25 ± 3
Mesentery	27 ± 4	20 ± 2	22 ± 0.2
Artera	20 ± 8	36 ± 0.5	24 ± 1.5
Lens	19 ± 1	9 ± 1	7 ± 2
Skin	10 ± 2	10 ± 1	13 ± 2
Liver	9 ± 0.5	13 ± 1	14 ± 2
Cornea	7 ± 2	7 ± 0.5	4.5 ± 1
Bone	7348 ± 83	8009 ± 145	7871 ± 14
Bovine adult platelets	223 ± 3	0	167 ± 0.5

*Values are given in ng/100 μg of total proteins. Each value is the mean (±SD) of triplicate measurements on one sample for RIA and duplicate measurements on one sample for IRMA.

difference between platelet and bone SPARC/Osteonectin could be observed in hydroxyapatite binding (72.5% ± 0.75 for platelet SPARC/Osteonectin and 62.8% ± 1.4 for bone SPARC/Osteonectin without BSA). This binding was highly specific as a concentration of 2 mg/ml BSA was necessary to inhibit it (for bovine bone and human platelet SPARC/Osteonectin, hydroxyapatite binding is respectively 55.1% ± 0.3 and 59.8% ± 3 for 0.2 mg/ml of BSA and 11.4% ± 0.3 and 11.2% ± 1 for 2 mg/ml of BSA).

Discussion

In this study, we have screened nonmineralized tissues from bovine fetus and platelets, with four Mabs recognizing four distinct epitopes of bovine bone SPARC/Osteonectin (Malaval et al. 1991). We show, by immunoblotting and IRMA, that platelet SPARC/Osteonectin presents a lower electrophoretic mobility and a lower affinity for Mab 2 than SPARC/Osteonectin present in bone as already described (Kelm et al. 1990; Malaval et al. 1991), but also in all nonmineralized tissues tested. When conditioned media and cell layer extract of the human osteosarcoma cell line MG 63, which has many characteristics of the osteoblast phenotype, were screened, the SPARC/Osteonectin detected presented the same electrophoretic reactivity and immunoreactivity pattern as bone SPARC/Osteonectin. These results suggest that the differences observed between "tissue type" SPARC/Osteonectin and the neo-secreted SPARC/Osteonectin from platelets do not result from an extracellular processing of the former, which is secreted as such. The 54 kD additional band observed on immunoblottings of cell layer extracts with Mab 3 (Fig. 2, lane B) presents a lower electrophoretic mobility than platelet SPARC/Osteonectin and might be the pre-protein described by Kuwata et al. (1985).

SPARC/Osteonectin from platelets, therefore, stands apart from the type found in all other tissues. Several hypothesis could be made about the structural differences existing between the two types of SPARC/Osteonectin. They include a difference in sequence, a variation in glycosylation, or other posttranslational modifications. Villareal et al. (1991) have recently established that the sizes of the mRNA for SPARC/Osteonectin produced in megakaryocytes and in a human osteosarcoma cell line SaoS 2 are identical, suggesting the identity of aminoacid sequences. The identity of proteolytic fragmentation of bone and platelet SPARC/Osteonectin has also been recently established (Kelm et al. 1991). This latter study also shows that the slightly lower electrophoretic mobility of platelet SPARC/Osteonectin compared to bone SPARC/Osteonectin is due to a difference in *N*-glycosylation between both proteins. Our data confirm that platelet SPARC/Osteonectin is deglycosylated by glycopeptidase F but not by endoglycopeptidase F, which excludes a high mannose or a biantennary complex-type glycosylation, while SPARC/Osteonectin from bone was deglycosylated by both enzymes, suggesting a high mannose or a biantennary complex-type glycosylation. We also observed that treatment of bone and platelets with glycopeptidase F suppresses the difference of electrophoretic mobility on SDS-PAGE gels. Recently, the immunoreactivity of Mab 2 with the expressed forms of two αgt 11 SPARC/Osteonectin cDNA clones was tested (Villareal et al. 1991b). Mab2 reacted with both transcripts, which localizes its epitope in the overlapping region of the clones (amino acids 18–146), and shows that it is not a carbohydrate epitope, the

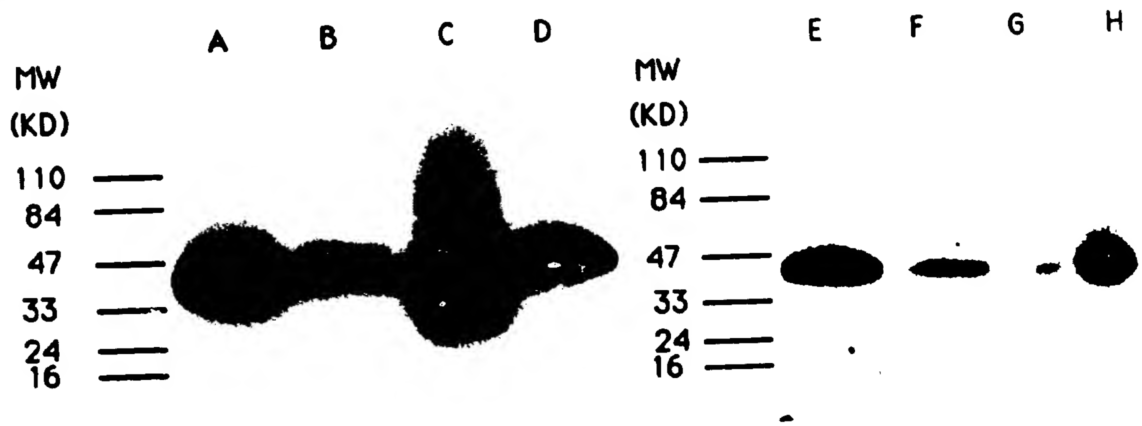

Fig. 2. Autoradiography of electroblots of conditioned media and whole cells lysates of MG63 osteosarcoma cells with anti-osteonectin monoclonal antibodies. (A), (E), and (H) Conditioned medium of human osteosarcoma MG63. (B), (F), and (G) Whole cells lysates of human osteosarcoma MG63. (C) Human bone extract. (D) Human platelet extract. Labeling antibodies were ^{125}I Mab3 (lanes A to D), ^{125}I Mab2 (lanes E and F), ^{125}K Mab6 (lanes G and H). Equal amounts of SPARC/Osteonectin were run in each lane as assessed by RIA (see methods). Gels were prepared and run according to the method of Schägger and Von Jagow (1987) under reducing conditions. Blots were performed according to Towbin et al. (1979). MW, Molecular weight standards.

Table II. Radioimmunoassay with four Mabs of glycosylated and deglycosylated bovine bone and human platelet osteonectin

Treatments	Tracers	Deglycosylation	Mab			
			Mab2	Mab3	Mab6	Mab12
Control	1251 BBO	No	30 ± 2	24 ± 2	28 ± 1	17 ± 1
Endo F		Yes	26 ± 1	19,5 ± 2	23 ± 0,5	11 ± 0,5
Endo F + inhibitor		No	35 ± 2	26 ± 1	33 ± 0,4	16 ± 0,3
PNGF		Yes	24 ± 0,2	23 ± 2	23 ± 16	14 ± 0,3
PNG F + inhibitor		No	32 ± 1	23 ± 1	27 ± 1	14 ± 1
Control	1251 HPO	No	5 ± 0,2	17 ± 1	21 ± 1	12 ± 1
Endo F		No	0,15 ± 0,8	12 ± 1	14 ± 17	6 ± 0,4
Endo F + inhibitor		No	0	14 ± 1	18 ± 0,2	8 ± 0,3
PNGF		Yes	0	11 ± 1	14 ± 1	5 ± 1
PNG F + inhibitor		No	1 ± 0,1	14 ± 1	15 ± 1	8 ± 0,3

Note: 1251 BBO; bovine bone osteonectin; 1251 HPO, human platelet osteonectin; Endo F, endoglycosidase F; PNG F, glycopeptidase F; Inhibitor, 1% SDS without Triton X100 in the assay buffer. Results are expressed as percent of total count. Each value is the mean (±SD) of triplicate measurements.

expressed proteins not being glycosylated. However, the epitope could be masked by a sugar chain. In the present work, the deglycosylation of SPARC/Osteonectin from bone and platelets did not affect the difference of immunoreactivity for Mab2 between these two proteins. This suggests that the reactivity with Mab2 is not related to the *N*-glycosylation of the molecule and that, if hampered accessibility is the origin of the reduced affinity for platelet SPARC/Osteonectin, it has to be due to another posttranslational modification. O-glycosylation of SPARC/Osteonectin seems to be excluded because SPARC/Osteonectin does not have O-glycosylation sites on its amino acid sequence.

Phosphorylation and sulfation are two other possibilities. According to the consensus features for tyrosine sulfation (Huttner & Baeuerle 1988), on the seven tyrosines present in the sequence of SPARC/Osteonectin, there is one possible sulfation site on tyrosin 162. However, presence of bound sulfate in SPARC/Osteonectin has never been documented. A recent work (Nagata et al. 1991) clearly showed that SPARC/Osteonectin is not sulfated in fetal rat calvarial cells in culture. Similarly, in an in vitro study of mouse osteoblasts labeled with ^{35}S-Sulfate, osteopontin, and bone sialoprotein seem to be the only sulfated proteins to be synthesized (Escarot-Charrier et al. 1989). A posttraductional modification of SPARC/Osteonectin by phosphorylation is more controversial. Several works have described the presence of bound phosphate on bovine and mouse SPARC/Osteonectin (Termine et al. 1981b; Romberg et al. 1984; Hugues et al. 1987). However, this protein did not incorporate $^{32}PO_4^-$ when fetal rat (Nagata et al. 1991) and porcine (Domenicucci et al. 1988) cal-

Fig. 3. Autoradiography of iodinated bovine bone osteonectin or human platelet osteonectin incubated with endoglycosidases. Lanes A to E: Iodinated bovine bone osteonectin. Lane A: Control. Lane B: Incubation with endoglycosidase F (0.05 U). Lane C: Inhibition of endoglycosidase F activity by 1% SDS. Lane D: Incubation with glycopeptidase F (1 U). Lane E: Inhibition of glycopeptidase F activity by 1% SDS. Lanes F–J: Iodinated human platelet osteonectin. Lane F: Control. Lane G: Incubation with glycopeptidase F (1 U). Lane H: Inhibition of glycopeptidase F activity by 1% SDS. Lane I: Incubation with endoglycosidase F (0.05 U). Lane J: Inhibition of endoglycosidase F activity by 1% SDS. Gels were prepared and run according to Schagger and Von Jagow (1987) under reducing conditions (see Methods). MW: Molecular weight standards.

varial cells, as well as bovine endothelial cells (Sage et al. 1984; 1989), were labeled in vitro.

The unique characteristics of SPARC/Osteonectin from platelets may be related to specific properties and roles. SPARC/Osteonectin purified from bone and platelets binds to thrombospondin with the same affinity (Clezardin et al. 1988). However, platelet SPARC/Osteonectin did not bind to types I, II, and V collagens in a solid phase assay by opposition to bone SPARC/Osteonectin (Kelm et al. 1991). The relationship between these differences in binding affinity and the structural and immunological differences previously described has to be established. In this study, we show that SPARC/Osteonectin from human platelets and bovine bone binds equally to hydroxyapatite. Interaction with hydroxyapatite is likely to occur in the acidic region of the amino-terminal portion of the molecule (domain I), which has been partly sequenced and found to be identical in bone and platelet SPARC/Osteonectin (Malaval et al. 1991; Kelm et al. 1990).

A growing number of experimental data suggests a role for SPARC/Osteonectin in cell/substrate and/or cell/cell interactions. Sage et al. (1989) have shown that SPARC/Osteonectin added to bovine aortic endothelial cell cultures induces changes in cell shape with a partial detachment of a confluent monolayer and inhibition of newly plated cells; work with synthetic peptides suggests that these properties can be ascribed to the first (I) and last (IV) domains of the protein (Lane & Sage 1990). The same team recently reported, with the same cell type, an inhibition of the entry into S phase (Funk & Sage 1991) and a stimulation of the expression of an inhibitor of tissue plasminogen activator (PAI-1) (Hasselar et al. 1991) induced by SPARC/Osteonectin. These results led to the suggestion that SPARC/Osteonectin may facilitate cell movements by interacting with the cell surface, either directly or in cooperation with other proteins. SPARC/Osteonectin has been shown to bind to platelets (Kelm et al. 1990; Clezardin et al. 1991). In a recent study we have shown that an antiserum raised against bovine bone SPARC/Osteonectin reduces the binding of thrombospondin to the platelets and inhibits collagen-induced platelet aggregation (Clezardin et al. 1991), suggesting a role for SPARC/Osteonectin in haemostasis, perhaps in cooperation with thrombospondin.

Further studies are necessary to assess whether the structural specificities of platelet SPARC/Osteonectin reflect specific functions. These studies will include the epitope mapping of our Mabs and the clarification of the structural requirements for the binding of Mab2.

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Advances in Brief

Quantitative Analysis of Circulating Tumor Cells in Peripheral Blood of Osteosarcoma Patients Using Osteoblast-specific Messenger RNA Markers: A Pilot Study[1]

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Abstract

Metastasis is a major cause of mortality and morbidity in osteosarcoma (OS) patients. To monitor tumor dissemination, we assessed the circulating tumor burden in OS patients by semiquantitative reverse transcription-PCR using osteocalcin, osteonectin, osteopontin, and type I collagen (*COLL*) mRNAs as molecular markers. We distinguished levels of the mRNAs in peripheral blood between OS patients and healthy subjects using an OS-derived cell line (Saos-2) as a reference standard. We prospectively analyzed 40 peripheral blood samples from 11 OS patients at diagnosis and 29 healthy subjects. In all 29 (100%) healthy subjects, we detected osteocalcin, osteonectin, and osteopontin mRNAs that were most likely attributed to illegitimate transcription in normal hematopoietic cells. In contrast, we found low *COLL* mRNA levels in only 35% (10 of 29) of healthy subjects, but significantly higher *COLL* mRNA levels in 91% (10 of 11) of OS patients ($P < 0.0001$). The reverse transcription-PCR assay for *COLL* mRNA was sensitive down to the detection of 10 Saos-2 cells among 10^6 normal peripheral blood nucleated cells. The upper limit of *COLL* mRNA determined among the healthy subjects was found exceeded by six OS patients. The substantially elevated *COLL* mRNA levels in peripheral blood seemed to originate from circulating malignant cells in these six OS patients, all of whom subsequently developed clinical metastases within 12 months of diagnosis ($P = 0.002$). Conversely, no metastases were detected in the remaining OS patients with normal *COLL* mRNA levels. Quantification of *COLL* mRNA may prove valuable for diagnosing OS micrometastasis and assessing prognosis.

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Introduction

OS[3] is potentially a fatal malignancy affecting predominantly children and young adults, where alterations of *Rb*, *p53*, *mdm2*, and *myc*, and *erbB-2* overexpression have been identified (1–5). However, the etiology and molecular mechanisms of OS remain unclear. Metastasis that occurs early in the natural history of OS is a major cause of mortality and morbidity. Virtually all OS patients may develop subclinical micrometastasis at initial diagnosis. Using computerized tomography scans, about 20% of OS patients have clinically detectable lung metastasis at presentation with consequently poor prognosis (6). Despite surgical resection of the primary lesion, nearly 90% of OS patients develop metastasis/recurrence after operation (7). Early detection of micrometastasis, or the potential for metastasis/recurrence, may permit prognostication and early treatment (8–10). In this regard, RT-PCR could be clinically valuable for the detection of micrometastasis or circulating malignant cells in OS patients.

OS produces osteoid and/or bone (11). We, therefore, selected molecular markers for OS based on the fact that *OC*, *ON*, *OPN*, and *COLL* mRNAs are differentially expressed in osteoblasts (12–15). *OC* is a bone matrix protein required for bone resorption, tissue remodeling, and extracellular matrix mineralization (12, 16). *ON* is a glycoprotein involved in extracellular matrix remodeling, cell adhesion, differentiation, and proliferation (17). Of interest, *ON* is also expressed in stromal myofibroblasts of carcinomas, at the interface between stromal cells and hepatocellular carcinoma cells, in melanoma cells, breast cancer, and colorectal cancer cells (18–22). Moreover, there is evidence suggesting that both *ON* and *OPN* are involved in angiogenesis and tumor progression (23, 24).

OPN is a bone matrix glycoprotein that modulates mineralization and bone resorption (14, 25, 26). Intriguingly, *OPN* mRNA is also expressed in human carcinomas, including breast, kidney, and endothelial cancers, where *OPN* may have adhesion/migration functions in promoting invasion and metastasis (27–30). In the circulation of patients with metastatic cancers, elevated *OPN* levels have been detected (31). Also, it has been demonstrated that tumor-derived *OPN* may enhance tumor growth and survival of metastases (32). *COLL* is another bone-specific marker gene, which encodes the major extracellular matrix component in bone (13).

In this prospective study, we evaluated whether *OC*, *ON*, *OPN*, and *COLL* mRNAs could be applied as molecular markers for detecting circulating tumor cells in peripheral blood of OS

[3] The abbreviations used are: OS, osteosarcoma; RT-PCR, reverse transcription PCR; *OC*, osteocalcin; *ON*, osteonectin; *OPN*, osteopontin; *COLL*, type I collagen; PBNC, peripheral blood nucleated cell; β-*2M*, β-2-microglobulin.

patients. Previous findings suggest that illegitimate transcription in normal hematopoietic cells may limit the specificity of RT-PCR (33, 34). To minimize this potential problem, we applied a quantitative approach for the precise assessment of the circulating tumor burden and, hence, the risk for metastasis/recurrence (8, 34). We, thus, developed a semiquantitative RT-PCR method for measuring levels of *OC*, *ON*, *OPN*, and *COLL* mRNAs in peripheral blood from OS patients and healthy subjects and correlated the mRNA levels with clinical outcomes of patients.

Materials and Methods

Peripheral Blood Samples from Patients and Controls. With informed consent and approval from the Ethics Committee of the Chinese University of Hong Kong, 40 peripheral blood samples were collected from 11 OS patients without clinically detectable metastases at diagnosis (median age, 21 yr; range, 12–37 yr; male:female ratio, 8:3) and 29 healthy volunteers between 19 and 40 yr of age. The diagnosis of all OS cases was histologically confirmed. The sites of primary OS were located in the femur, proximal tibia, proximal humerus, and pelvis. All of the patients were treated by surgery and chemotherapy and were followed up clinically for at least 12 months. The healthy subjects served as negative controls for semiquantitative RT-PCR.

PBNC Isolation, RNA Extraction, and DNase I Digestion. PBNCs were isolated by Ficoll-Paque (Pharmacia Biotech, Uppsala, Sweden) from 20 ml of citrated blood from the OS patients and healthy subjects studied. After washing in 30 ml of PBS and centrifugation at 100 × *g* for 10 min, the cell pellet was resuspended in 1 ml of PBS. The number of PBNCs was counted in a hemocytometer. After centrifugation, the cell pellet was resuspended in 0.5 ml of guanidinium thiocyanate solution and total RNA was extracted by a single-step method (35). Before RT-PCR, total RNA was treated with DNase I to remove contaminating genomic DNA. Digestion was conducted at 37°C for 1 h in the presence of 10 units of DNase I (Boehringer Mannheim, Mannheim, Germany), 10 mM $MgCl_2$, 0.1 mM DTT, and 50 mM Tris-HCl. After heat-inactivation, RNA was extracted using phenol-chloroform and then ethanol-precipitated.

Cell Culture. An OS-derived cell line, Saos-2 (American Type Culture Collection, Manassas, VA), was used to establish standard curves for measuring levels of the mRNA markers. The cell line was cultivated in DMEM added with penicillin, streptomycin, 2 mM glutamine, and 10% fetal bovine serum (Life Technologies, Inc., Gaithersburg, MD). The medium was changed every 3 days, and the cells were harvested when the growth was subconfluent. The total number of Saos-2 cells was counted in a hemocytometer.

Development of Standard Curves Using the Saos-2 Cell Line. To simulate the presence of OS cells in the circulation of OS patients, total RNA was first extracted from 10^7 normal PBNCs and 10^7 OS cells from the Saos-2 cell line. Aliquots of total RNA from 10^6 normal PBNCs were mixed with Saos-2 total RNA, corresponding to 1, 10, 10^2, 10^3, 10^4, 10^5, and 10^6 tumor cells (based on the calculation of the average amount of RNA extracted per cell). The RNA mixtures were subjected to semiquantitative RT-PCR.

Table 1 Sequences of sense (*F*) and antisense (*R*) primers for RT-PCR and oligonucleotide probes (*P*) for Southern blot analysis

Primer/probe	Sequence
OCF	5′-TGCAGAGTCCAGCAAAGGTGCA-3′
OCR	5′-ATAGGCCTCCTGAAAGCCGATGT-3′
ONF	5′-GATCTTCTTTCTCCTTTGCCTGG-3′
ONR	5′-TGTTTGCAGTGGTGGTTCTGGCA-3′
OPNF	5′-TCACAGCCATGAAGATATGCTGG-3′
OPNR	5′-TACAGGGAGTTTCCATGAAGCCAC-3′
COLLF	5′-GGTGGTGGTTATGACTTTGGTT-3′
COLLR	5′-CTTGGCTGGGATGTTTTCAGGT-3′
COLLP	5′-ATAGTGCATCCTTGGTTAGGGTCAATCCAG-3′
β-*2MF*	5′-CCTGAATTGCTATGTGTCTGGGTTTCATCCA-3′
β-*2MR*	5′-GGAGCAACCTGCTCAGATACATCAAACATGG-3′

Semiquantitative RT-PCR and Southern Blot Analysis. Total RNA (1 μg) was denatured at 65°C for 2 min and annealed with 1 μg of random primers at 37°C for 10 min (34). RT was carried out in 1× reaction buffer [50 mM Tris-HCl (pH 8.3), 75 mM KCl, and 3 mM $MgCl_2$] with 10 mM DTT, 0.5 mM deoxynucleoside triphosphates, and 0.5 μl of RNase block (Stratagene, La Jolla, CA). cDNA was synthesized at 37°C for 1 h using 200 units of Moloney murine leukemia virus reverse transcriptase (Life Technologies, Inc.). The reaction was stopped at 70°C for 7 min.

PCR amplification of *OC*, *ON*, *OPN*, and *COLL* cDNAs was conducted using gene-specific primers lying within different exons to give products of 199 bp, 225 bp, 298 bp, and 325 bp, correspondingly (Table 1). β-*2M* mRNA served as an internal control to ensure that an exact amount of high-integrity total RNA was reverse-transcribed to produce cDNA in each assay (34, 36).

PCR was conducted in 1× PCR buffer [20 mM Tris-HCl (pH 8.4), 50 mM KCl, and 2.5 mM $MgCl_2$] added with 0.2 mM deoxynucleoside triphosphates; 30 pmol of sense and antisense primers for *OC*, *ON*, *OPN*, *COLL*, or β-*2M* cDNA; 3 μl of cDNA; and 2.5 units of Taq DNA polymerase (Life Technologies, Inc.; Ref. 34). The optimized thermal profile was initiated with a 5-min denaturation at 94°C, followed by 30 cycles of 94°C for 1 min, 61°C (β-*2M*), 69°C (*OC/ON*), 65°C (*OPN*), or 67°C (*COLL*) for 1 min and 72°C for 1 min, and a final extension at 72°C for 10 min. Aerosol-resistant pipette tips and separate areas were used for pre-PCR, PCR, and post-PCR procedures. Each sample was analyzed in duplicate. Saos-2 RNA standards and multiple water blanks were analyzed in parallel with peripheral blood samples for each set of PCR. PCR products were loaded onto 2% agarose gels and stained with ethidium bromide.

The gene-identity of the PCR product was verified by nonradioactive Southern blot analysis using a gene-specific oligonucleotide (Table 1), which was labeled at the 3′ end with digoxigenin (34, 36). Chemiluminescent detection was conducted using disodium 3-(4-methocyspiro{1, 2-dioxetane-3,2′′-(5′′-chloro) tricyclo[3.3.1.1^{3,7}] decan}-4-yl) phenyl phosphate (Boehringer Mannheim). By using imaging densitometry (Bio-Rad, Hercules, CA), the amounts of PCR products for blood samples were quantified on the same Southern blot as the PCR products generated for establishing the Saos-2 standard curve.

Table 2 Levels of *OC*, *ON*, *OPN*, and *COLL* mRNAs in PBNCs from 29 healthy subjects

	OC	*ON*	*OPN*	*COLL*
mRNA levels	High	High	High	Low
RT-PCR positivity	29	29	29	10
% of positivity	100%	100%	100%	35%

Results

Levels of Osteoblast-specific mRNAs in PBNCs from Healthy Subjects. *OC*, *ON*, and *OPN* mRNAs were detected in PBNCs from all 29 (100%) healthy subjects at levels undistinguishable from those in the Saos-2 cell line (Table 2). In contrast, *COLL* mRNA levels, markedly lower than in Saos-2 cells, were obtained in only 35% (10 of 29) of healthy subjects (Fig. 1*A*). The frequency of *COLL* mRNA detection among the control subjects was much lower than that for *OC*, *ON*, and *OPN* mRNAs. We, therefore, determined the upper limit of *COLL* mRNA in the control group (mean + 3 SD) and set this as the reference range for distinguishing *COLL* mRNA levels in circulation between OS patients and healthy subjects.

Linear Saos-2 Standard Curve for *COLL* mRNA Measurement. A linear relationship was found between the amount of *COLL* PCR product and the level of Saos-2 total RNA used corresponding to 0.13 ng to 1.3 μg, over a range of $10-10^5$ Saos-2 cells (correlation coefficient, 0.94; Fig. 2). The RT-PCR assay was consistently sensitive down to the detection of 10 Saos-2 cells among 10^6 normal PBNCs.

Quantification of *COLL* mRNA in Peripheral Blood from OS Patients and Association with Clinical Metastases. In this prospective study, we detected variable levels of *COLL* mRNA in 20 ml of peripheral blood from 91% (10 of 11) of OS patients at diagnosis (Fig. 1*B* and Fig. 3). The *COLL* mRNA levels in the OS patients were significantly higher than in the 29 healthy subjects studied (Mann-Whitney *U* test, $P < 0.0001$). According to the Saos-2 standard curve, the mean *COLL* mRNA level in the control group ($n = 29$) was 767.5 Saos-2-RNA equivalents (pg; range, 0.1−5338 Saos-2-RNA equivalents; Fig. 3). The mean *COLL* mRNA level among the 11 OS patients was 36361.95 Saos-2-RNA equivalents (pg; range, 1.7−132373.3 Saos-2-RNA equivalents; Table 3). The upper limit of *COLL* mRNA (mean + 3 SD) in the control group (5338 Saos-2-RNA equivalents) was found exceeded by six OS patients (Fig. 3).

Of clinical relevance, all of the six OS patients with substantially raised *COLL* mRNA levels subsequently developed metastases within 12 months of diagnosis (Table 3). Two of the six patients with the highest *COLL* mRNA levels developed clinical metastases within 4−5 months of diagnosis, whereas the other four patients with similarly high *COLL* mRNA levels developed metastases within 6−10 months of diagnosis. In striking contrast, no metastases were detected in any of the remaining five OS patients with normal *COLL* mRNA levels ($P = 0.002$; Fisher's exact test).

Discussion

In this first attempt to detect circulating malignant cells in peripheral blood of OS patients, we have developed semiquantitative RT-PCR for *COLL* mRNA using the Saos-2 cell line as a reference standard. As compared with healthy subjects, markedly elevated *COLL* mRNA levels in peripheral blood from OS patients at diagnosis were strongly associated with the subsequent development of clinical metastases ($P = 0.002$). On the other hand, the presence of *OC*, *ON*, and *OPN* mRNAs in PBNCs from all of the healthy subjects studied was most likely attributed to illegitimate transcription in normal hematopoietic cells. These results suggest that *OC*, *ON*, and *OPN* mRNAs are not specific markers for OS. Our present data are consistent with the fact that *OC* mRNA is expressed in peripheral blood platelets, bone marrow megakaryocytes, and multiple soft tissues such as aorta, liver, lung, kidney, and brain (37, 38). Moreover, *ON* mRNA has been detected in developing blood vessels, and its transcription can be induced by transforming growth factor β (23, 39, 40). Furthermore, *OPN* mRNA expression in macrophages has been documented, and its transcription is inducible by transforming growth factor β or during the activation of natural killer cells (19, 26, 29, 41).

Using RT-PCR with DNase I pretreatment, we demonstrated low levels of *COLL* mRNA in PBNCs in only 35% (10 of 29) of healthy subjects. Because the PCR product has the expected molecular size, the possibility of genomic DNA contamination can be ruled out. Because *COLL* mRNA is also expressed in skin (42, 43), the first aliquot of peripheral blood might have skin contamination caused by needle aspiration. We may be able to eliminate this kind of contamination by disregarding the first aliquot of blood and collecting subsequent aliquots for molecular analysis. However, the low levels of *COLL* mRNA detected in healthy subjects were also highly likely attributed to illegitimate transcription in normal hematopoietic cells. To minimize this potential problem, we have applied a quantitative approach for differentiating the *COLL* mRNA levels in peripheral blood between OS patients and healthy subjects. Our semiquantitative RT-PCR enables us to determine the upper limit of *COLL* mRNA among healthy subjects. Above this reference range, elevated *COLL* mRNA levels (up to ~25-fold) in 55% (6 of 11) of OS patients at diagnosis may genuinely reflect the presence of circulating malignant cells in complete concordance with the subsequent development of clinical metastases.

Unlike DNA alterations such as *p53* and *Rb* mutations, which were inconsistently found in OS and micrometastasis (1, 2), markedly raised *COLL* mRNA levels were frequently detectable among the OS patients studied. Moreover, the semiquantitative RT-PCR assay that measures mRNA levels offers much higher sensitivity than mutation screening by DNA sequencing. As opposed to oncogene/tumor suppressor gene alterations that could also be found in a wide variety of other tumors, the advantage of using *COLL* mRNA as a marker for detecting OS micrometastasis is its relatively high osteoblast specificity.

For OS may have already metastasized at clinical presentation, early detection of micrometastasis is critical for permitting early chemotherapy or intensive adjuvant chemotherapy, which should be more effective against micrometastasis than clinically detectable metastasis (7). In our cohort, all of the six (100%) OS patients with substantially elevated *COLL* mRNA levels subsequently developed clinical metastases within 12

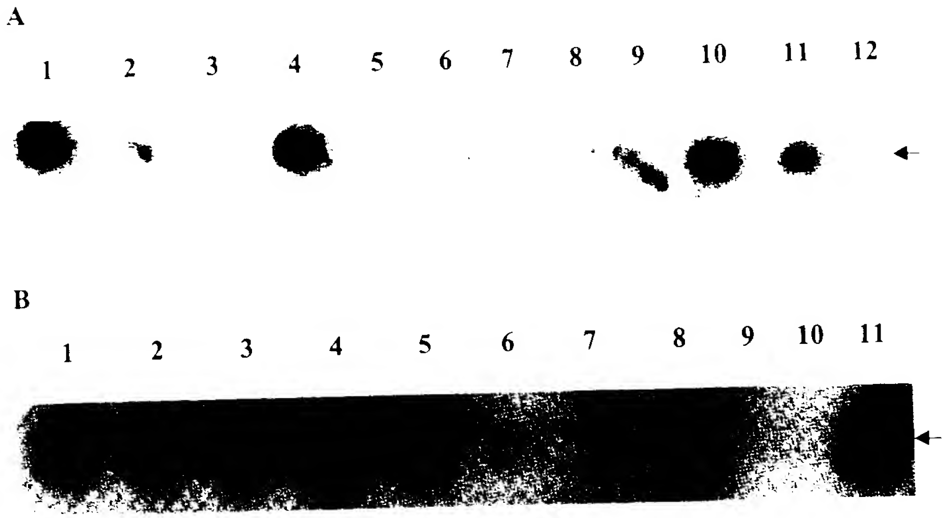

Fig. 1 Semiquantitative RT-PCR for *COLL* mRNA in peripheral blood and Southern blot analysis. *A*, healthy subjects (*Lanes 1–12*). *B*, OS patients (*Lanes 1–11*).

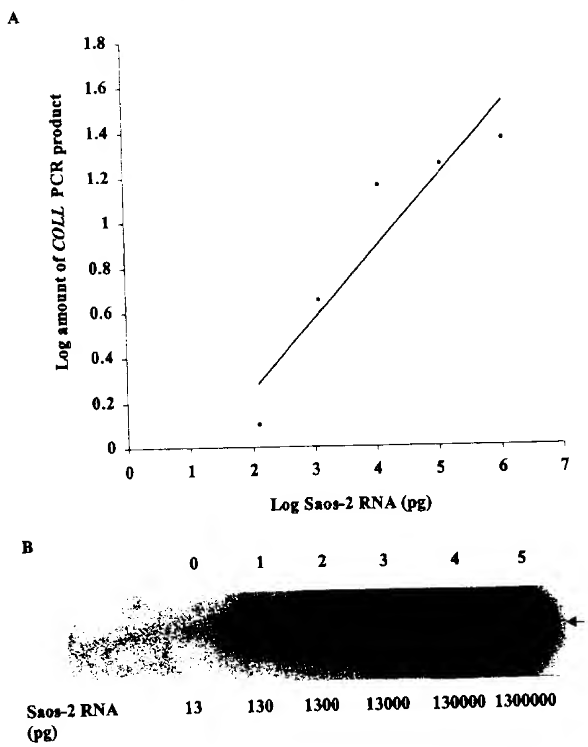

Fig. 2 Linear relationship between the amount of *COLL* PCR product and the level of Saos-2 total RNA used for semiquantitative RT-PCR (on logarithmic scales). *A*, the equation of linear regression is $y = 0.313x - 0.381$ (correlation coefficient, 0.94). *B*, semiquantitative RT-PCR for *COLL* mRNA and Southern blot analysis using 13 pg to 1.3 μg of Saos-2 RNA, over a range of $1-10^5$ Saos-2 cells, as shown in *Lanes 0–5*.

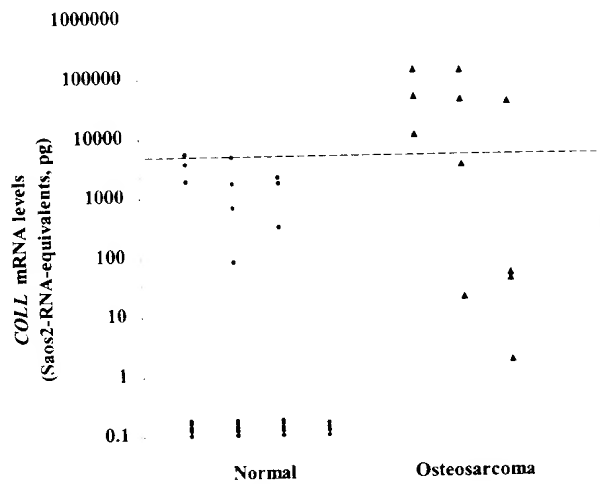

Fig. 3 Levels of *COLL* mRNA in 40 peripheral blood samples from 29 normal subjects and 11 OS patients with reference to Saos-2-RNA equivalents (pg). The upper limit of *COLL* mRNA (mean + 3 SD) in the normal control group (5338 Saos-2-RNA equivalents) is marked with a *dotted line*.

months of diagnosis. This strongly suggests that *COLL* mRNA may be applied as a prognostic marker to identify OS patients at diagnosis with a high risk of metastasis/recurrence; such disease progression could possibly be prevented by early treatment. Because the most frequent site of metastasis is the lung (6), the sputum from OS patients would possibly be another sample source to be tested for circulating OS cells using semiquantitative RT-PCR for *COLL* mRNA.

Furthermore, the *COLL* mRNA quantity in PBNCs may provide useful diagnostic information in conjunction with cross-sectional imaging results before histological confirmation can be made on surgically resected tumors/biopsies. Characterization of circulating OS cells detected might also contribute to the understanding of the OS pathogenesis. For further investigation, it is worthwhile to explore the diagnostic and prognostic significance of the *COLL* mRNA level in a larger series of OS patients with long-term follow-up.

As a potential prognostic factor, the *COLL* mRNA level in peripheral blood may be sequentially monitored to follow up OS patients without micrometastasis at diagnosis. For the five OS

Table 3 Patient characteristics and the association of *COLL* mRNA levels in PBNCs measured at initial diagnosis with clinical outcomes within 12 months of diagnosis

Patient	Sex/Age	Site of primary OS	*COLL* mRNA level in PBNCs (Saos-2-RNA equivalents, pg)	Clinical evidence of metastasis (months after initial diagnosis)
OS1	M/17	Femur	10,510.41	Yes (9)
OS2	F/12	Proximal tibia	3,367.62	No
OS3	M/16	Humerus	47,128.71	Yes (8)
OS4	M/15	Humerus	132,373.33	Yes (5)
OS5	M/18	Femur	40,640.46	Yes (6)
OS6	M/25	Pelvis	40.20	No
OS7	M/21	Femur	49.80	No
OS8	M/37	Humerus	37,631.50	Yes (10)
OS9	F/23	Proximal tibia	20.10	No
OS10	M/24	Pelvis	1.70	No
OS11	F/28	Proximal tibia	128,217.60	Yes (4)

patients studied without evidence of micrometastasis, as reflected by normal *COLL* mRNA levels in PBNCs, the toxicity of chemotherapy might be avoided at an initial stage (7). During clinical follow-up, quantification of *COLL* mRNA in peripheral blood may help assess the patients' response to therapies, which is crucial in determining the patients' prognosis. The molecular approach of using *COLL* mRNA may possibly open up the prospect of monitoring OS in a noninvasive manner without the requirements of surgery and computerized imaging. As compared with these conventional methods, semiquantitative RT-PCR for *COLL* mRNA seems to be sufficiently sensitive, rapid, and reliable for evaluating histological and tumor response to treatments. Taken together, this novel mRNA marker could potentially help manage OS patients more effectively and, hence, improve the clinical outcome or guide the selection of therapies.

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Effect of different Ti–6Al–4V surface treatments on osteoblasts behaviour

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Abstract

The purpose of the present work was to examine the effect of different Ti–6Al–4V surface treatments on osteoblasts behaviour. Previous work in this laboratory has demonstrated that an ageing treatment reduces metal ion release from this alloy compared to standard passivation procedures. In this study, human osteosarcoma MG-63 were used in short-term in vitro tests to assay for cell viability and cell proliferation at 12, 24 and 72 h while SaOS-2 were used in long-term in vitro tests to assay for osteonectin, osteopontin, osteocalcin gene expression, total protein amount (TP), alkaline phosphatase activity (ALP) and fibronectin production (FN) for 1–4 weeks. Epifluorescence microscopy was used to observe SaOS-2 cell morphology. After 24 h, there was no difference in MG-63 cell viability proliferation or in SaOS-2 cell morphology between the different surface treatments. For the long-term tests, the aged Ti–6Al–4V induced significantly higher cell proliferation than the control Ti–6Al–4V at 72 h. At week 1, no difference in the osteonectin, osteopontin, and osteocalcin gene expression was found between samples. The peak of ALP activity appeared earlier at week 2 for the control surface compared with the passivated and aged surfaces. The early increase in ALP activity for the control sample could be a compensatory effect of decreased osteoblasts proliferation. There was no difference in the expression of FN for the different surface treatments. Our present results showed that the different surface treatments, which induced different metal ion release kinetics and surface properties, influenced the cell proliferation and ALP activity of osteoblast cells. Aluminium ions release kinetics as well as presence of vanadium ions may play a major role in influencing the osteoblasts behaviour in the present study.

Keywords: Ti–6Al–4V; Surface treatment; Metal ion release; Human osteosarcoma cells; Differentiation; Cell morphology

1. Introduction

The interaction between solid surfaces and biological systems are critically important to many areas of medicine, technology and research. In general, only the surface of an implant is in direct contact with the host tissue, and thus this portion of the material plays a central role in determining its biocompatibility. The surface of material can change with time, and is often distinctly different from the bulk properties, because of oxidation and contamination. Although the surface clearly plays an important role in implant cell interactions, the relationships between surfaces of the implant, its reactivity with tissue constituents, and long-term integrity and clinical efficacy are still poorly understood [1,2].

Ti–6Al–4V alloy has become one of the most used biomaterials due to its excellent corrosion resistance, good mechanical properties and low toxicity [3]. Its corrosion resistance is due to the oxide film, which forms spontaneously on exposure to air. However, when the titanium alloy is implanted into a complicated and aggressive physiological in vivo environment, the oxide stability may be affected, resulting in increased metal ion release [4]. Elevated levels of metal ions have been reported in the serum of patients with both well functioning and failed total joint replacements [5]. In addition, aluminium (Al) has well documented toxic effects in the serum or urine of patients who had a total

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hip replacement made of titanium alloy [6]. Several in vitro studies have been carried out with a variety of cell lines to test metal ion toxicity, which is suspected of playing a significant role in cell behaviour. Titanium ions have been shown to influence mineral formation and osteoid nodules in rat calvaria cultures [7]. It has been demonstrated that Ti 6Al 4V ion solution can affect the normal differentiation of bone marrow stromal cells to mature osteoblasts in vitro [8]. Therefore, decreasing the metal ion release could be a method to increase the biocompatibility of Ti alloy.

In our previous work, it has been shown that a simple thermal treatment, ageing in deionised distilled boiling water, improves the dissolution resistance of Ti 6Al 4V alloy compared to conventional passivation treatments [9]. In the present study, the biocompatibility of the aged surface was evaluated in comparison to the control and the passivation treatments. Short- and long-term in vitro tests were performed with two osteoblastic cell corresponding to immature and mature cells. The biocompatibility of the aged titanium alloy was evaluated in vitro with short-term tests assessing cell viability, cell proliferation and cell morphology. Then, quantification of the ageing treatment was performed with long-term in vitro tests quantifying osteonectin, osteopontin, osteocalcein gene expression, FN production, ALP activity and TP amount.

2. Materials and methods

2.1. Ti alloy surface preparations

Distal sections of forged Ti 6Al 4V alloy femoral stems from the Ti-Mod Freeman hip replacement were supplied by Finsbury Instruments (Leatherhead, Surrey, UK). The hip stems were cut into discs of 4 and 10 mm^2 and of 1 mm thickness. The samples were first wet-ground with 120, 600, 1200, 2400 and 4000 grit silicon carbide abrasive paper (Struers, UK) at approximately 150 rpm, then polished with 6, 3 and 1 μm diamond solution (Microcloth Buehler, UK) on a clean polishing cloth (Microcloth Buehler, UK) and finally with colloidal silica polishing suspension (0.06 μm, Mastermet Buehler, UK). Following polishing, the samples were cleaned in 1% Triton solution for 1 h and rinsed in deionised distilled water. This treatment was used as a control (C). The practice for surface preparation of surgical implants involves a nitric acid passivation treatment (P) based on the ASTM F86 protocol using 30% nitric acid for 1 h [10]. A third treatment (the ageing treatment, A) consisted of the passivated treatment followed by ageing in boiling deionised distilled water for 10 h. The successive steps of the three surface treatments are shown in Table 1. Finally, the discs were rinsed several times with sterile, endotoxin-free PBS before cells were seeded. As preliminary experiments, the endotoxin assay using Limulus Amebocyte Lysate (BioWhittaker, Emerainville, France) revealed that no contamination of lipopolysaccharides (LPS) was present on the surface of the treated samples.

2.2. Cell culture

Two human osteoblast-like cell lines obtained from American Tissue Culture Collection (Manassas, VA, USA) were tested: an immature osteoblast (MG-63) and a mature osteoblast (SaOS-2) [11]. Two cell lines were used as it has been shown that cellular response depends on the local environment as well as on cell state of maturation [12]. The osteoblasts were cultured in Dulbecco's modified Eagle's medium (DMEM) (Sigma, Buchs, Switzerland) containing 10% fetal bovine serum (Sigma), 1% PSF (100×, 10,000 U/ml Penicillin, 10,000 μg/ml Streptomycin and 25 μg/ml Fungizone®) (GibcoBRL, Life Technologies, Basel, Switzerland) under a humidified 5% CO_2 air atmosphere at 37°C.

MG-63 cells were seeded: (1) at a concentration of 5000 cells/well on 4 mm^2 samples (C, P, A) for cell proliferation assessment in 96 well cell culture plates;

Table 1
The successive steps of surface treatments for the control (C), the passivated (P) and the aged (A) Ti 6Al 4V surfaces

	Control (C)	Passivated (P)	Aged (A)
Surface preparation	Polished Cleaning in 1% Triton for 1 h Rinsing in deionised distilled water	Polished Cleaning in 1% Triton for 1 h Rinsing in deionised distilled water 30% nitric acid for 1 h Rinsing in deionised distilled water	Polished Cleaning in 1% Triton for 1 h Rinsing in deionised distilled water 30% nitric acid for 1 h Rinsing in deionised distilled water 10 h in boiling deionised distilled water
Sterilisation	Immersed in 100% ethanol for 10 min Air-dried Exposed under UV light for 30 min on each side Rinsing in endotoxin free phosphate buffered solution (PBS)		

(2) at a concentration of 100,000 cells/well on $10\,mm^2$ samples (C, P, A) for cell viability assessment in 24 well cell culture. Proliferation and viability measurements were performed at 12, 24 and 72 h. SaOS-2 cells were seeded at a concentration of 100,000 cells/well on $10\,mm^2$ samples (C, P, A) for quantification of osteonectin, osteopontin, osteocalcin gene expression at week 1 and ALP activity, FN and TP at week 1, 2, 3 and 4. The medium was changed every 3–4 days. For ALP, FN and TP measurements, the medium was removed two days before each time point (at week 1, 2, 3 and 4) and cells were washed 3 times with PBS to avoid the effects of serum composition on the biochemical assays. The cell culture was then incubated with serum free medium 2 days before biochemical assays.

2.3. *Cell viability/proliferation*

To assay cell viability, cells were collected by trypsinisation with 1× trypsin-EDTA solution (Sigma) 5 min. The collected cells were double stained with 25 μg/ml fluorescein diacetate (Sigma) and with 20 μg/ml propidium iodide (Sigma) in PBS for 5 min [13]. Live cells appear green and dead cells appeared red under epifluorescent illumination (blue filter of 450–490 nm and green filter of 510–560 nm which allowed visualisation of the green and red fluorescing cell, respectively). Cell viability tests were performed three times for each point and at least 200 cells were counted in the epifluorescent mode of a Nikon Microphot-FXA microscope (Nikon, Tokyo, Japan). Cell viability was defined as the ratio of viable cells to the total number of cells and presented in percentage (%) for the treatments (C, P, A). To assay cell proliferation, cells were treated with CellTiter 96® AQ$_{ueous}$ Assay (Promega Corp., WI, USA). The assay is based on the reduction of a tetrazolium compound to a coloured formazan product by viable cells (or metabolic activity). The absorbance at 490 nm is directly proportional to the cell proliferation. The cell proliferation of the passivated (P) and the aged (A) samples was normalised by the control (C).

2.4. *Osteonectin, osteopontin, osteocalcin gene expression*

Cell lysate was collected after week 1 and total RNA was isolated and purified with RNeasy columns (Qiagen, Basel). The isolated RNA was reversed transcripted to cDNA with the StratScript enzyme (Stratagene, San Diego, CA). Quantitative real time RT-PCR (TaqMan ABI Prism 7700, Applied Biosystem, Foster City, CA) was used to measure the gene expression of osteonectin, osteopontin, osteocalcin, and 18S using Amplifluor Universal Detection System (Intergen, Purchase, NY). Primers were designed with the Software Primer Express™ (Applied Biosystem). Primers were purchased from Integrated DNA Technologies (Coralville, IA). Use of a housekeeping gene (18S) allowed the different samples to be normalized and compared between experiments.

2.5. *Total protein*

A solution of 0.5 ml 1% Triton-X in MilliQ water was used to lyse cells. The lysate was sonicated for 30 s at 40 W. The total protein amount was measured in the cell lysate with DC (Detergent Compatible) Protein Assay Kit (Bio-Rad Lab, CA, USA).

2.6. *Alkaline phosphatase activity*

ALP activity is considered to reflect osteoblastic activity and is thought to play a major role in bone formation and mineralisation [14]. The enzyme activity within the lysate was measured with a commercial Kit (Sigma ALP-10, cat. no.245). One unit of LPA activity is defined as that amount of enzyme, which produces 1 μmol of *p*-nitrophenol (PNP)/min. The total protein amount was used to normalise the ALP activity.

2.7. *Fibronectin measurement*

FN is one of the most abundant extracellular matrix components in many tissues and has been shown to be present in early bone formation [15]. A commercially available Human Fibronectin ELISA kit (Biomedical Tech Inc., Stoughton, MA, USA) was used to measure FN within the lysate. The FN production was normalised by the total protein amount.

2.8. *Statistical analysis*

The viability and proliferation of MG-63 are presented at each time point (12, 24, and 72 h) corresponding to the mean ± standard error of three independent analyses. Gene expression results of SaOS-2 are presented at week 1 and corresponded to the mean ± standard error of three independent analyses performed in duplicates. The ALP activity and FN normalised by TP of SaOS-2 are presented at each time point (1, 2, 3 and 4 weeks) corresponding to the mean ± standard error of four independent analyses performed in duplicates. ANOVA was used to determine the statistical significance of the differences observed between groups. p values smaller than 0.05 were considered significant.

2.9. *Cell morphology*

Cell morphology studies can provide information concerning the cell interaction with the treated

Ti 6Al 4V implant and cell cell contact. SaOS-2 cells were seeded on to each sample (10 mm^2) at a concentration of 2000 cells well in 24-well tissue culture plates for 24 h and 7 days. Cells were then fixed with 1% glutaraldehyde (Sigma) in PBS, treated with 1% Triton X, 2 mg ml sodium borohydride (Sigma) in PBS and stained with 4 μg ml rhodamin-phalloidin (Sigma) for actin microfilaments. A Nikon Eclipse TE300 inverted epifluorescence microscope enabled cells to be visualised. A Standard filter set (510 nm) was used for rhodamin-phalloidin. Images were acquired with a Micromax PB1300 cooled CCD camera (Roper Scientific, Trenton, NJ) and image contrast enhancing was performed using a MetaMorph imaging system (Universal Imaging Corporation, Westchester, PA). The cell morphology and particularly the actin filament organisation were visually examined.

3. Results

3.1. Cell viability proliferation

There was no significant difference between the three different treated samples for the cell viability of MG-63 during the test (Fig. 1). The aged sample had higher cell proliferation compared to the passivated samples at 72 h (Fig. 2).

3.2. Osteonectin, osteopontin, osteocalcin gene expression

There was no significant difference between the three different treated samples for the osteonectin, osteopontin, and osteocalcin gene expression of SaOS-2 at week 1 (Fig. 3). The level of gene expression was comparable for osteonectin and osteopontin for each sample while higher level was found for osteocalcin of MG-63 during the test (Fig. 1).

3.3. Alkaline phosphatase activity

The ALP activity of the control sample was statistically higher than the passivated and the aged samples at week 2 (Fig. 4). At week 4, the aged sample induced a statistically higher ALP activity than the passivated and the control samples.

3.4. Fibronectin measurement

No FN activity was detected at week 1 (Fig. 5). FN activity was observed after week 2 and reached a peak at

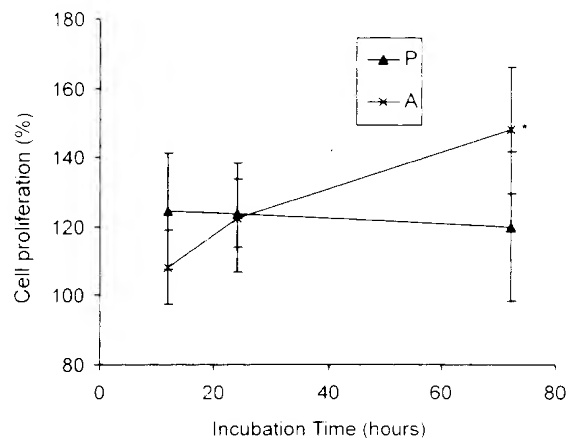

Fig. 2. Proliferation of MG-63 cells on the different Ti 6Al 4V samples. The proliferation of P and A was normalised by the proliferation of C. A significant difference between the aged and passivated samples was found at 72 h (*: $p \leq 0.05$; P, passivated; A: aged).

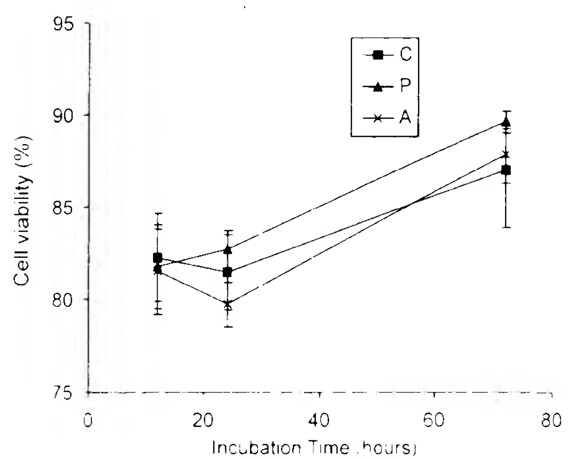

Fig. 1. Viability of MG-63 cells on the different Ti 6Al 4V samples. There was no significant difference between the three different treated samples (C control, P passivated, A aged) for the MG-63 cell viability at 12, 24 and 72 h.

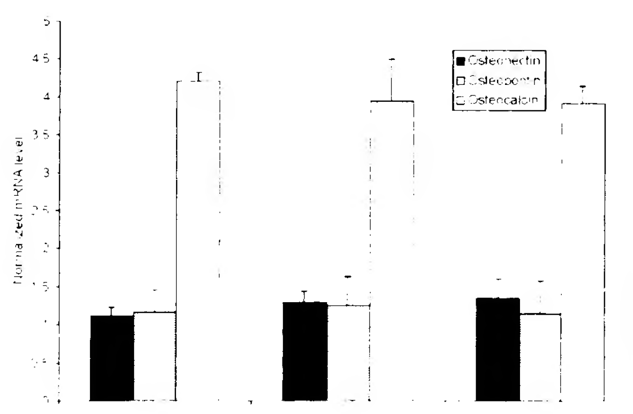

Fig. 3. Gene expression of osteonectin, osteopontin, and osteocalcin normalised by the gene expression of 18S on the different Ti 6Al 4V samples. There was no significant difference between the three different treated samples (C control, P passivated, A aged) for the SaOS-2 cells gene expression at week 1.

week 3 before decreasing at week 4. There was no significant difference among the three surface treatments.

3.5. Cell morphology

After 24 h, bundles of actin filaments (stress fibres) were found and cells had an angular shape on the three different treated surfaces. Focal adhesion and some small stress fibres were also visible at the cells periphery (Fig. 6). After 7 days, cell–cell contact was visualised for the osteoblasts on these three surface treatments. No major difference was visually found between the three surface treatments.

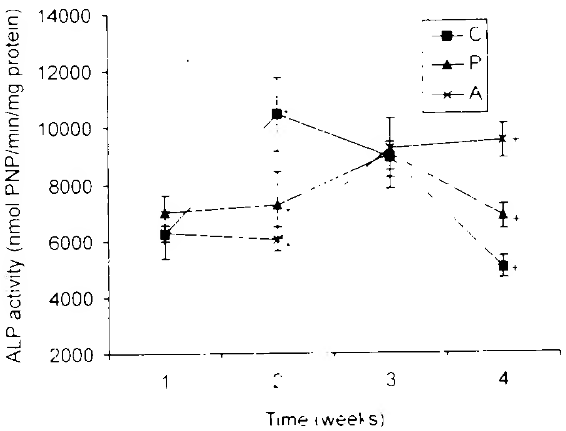

Fig. 4. ALP activity of SaOS-2 cells on the treated Ti–6Al–4V samples (C: control, P: passivated, A: aged). At week 2, there was a significant difference between C versus P and between C versus A (* $p \leq 0.05$). At week 4, there was a significant difference between C versus A and between P versus A (* $p \leq 0.05$).

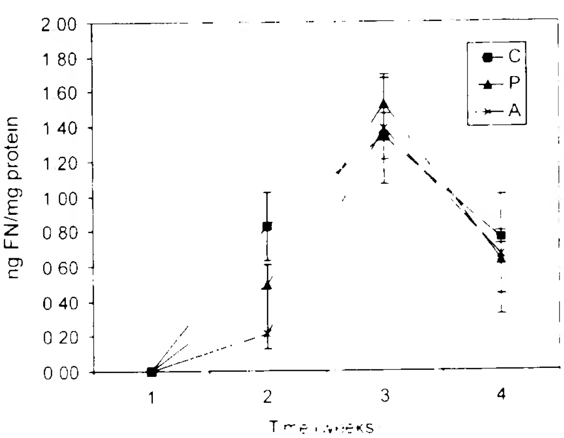

Fig. 5. Fibronectin measurements of SaOS-2 cells on the different Ti–6Al–4V samples. No fibronectin was detected for all samples at week 1. There was no significant difference between the three different treated samples (C: control, P: passivated, A: aged) for the SaOS-2 cells fibronectin production at week 2, 3 and 4.

4. Discussion

In short-term in vitro tests (24 h), cell viability, proliferation and cell morphology demonstrated no evidence of cell toxicity in response to the different treated Ti–6Al–4V surfaces. Moreover, the cell morphology of SaOS-2 clearly showed that focal adhesion sites localised at the periphery and bundles of actin filaments spanned the entire cells when cells were seeded on the three different samples (Fig. 6). Based on these results, it was not possible to discriminate the effects of the different surface treatments on osteoblasts behaviour.

Differences between osteoblasts behaviour became apparent only since 72 h with a significant difference in proliferation between the aged and the passivated samples (Fig. 2). The proliferation measurement was based on cellular reduction of MTS and was dependent on the reduced pyridine nucleotides NADH and NADPH, i.e. finally on mitochondrial activity. The cellular damage in mitochondria inevitably results in loss of the ability of the cell to maintain and provide energy for metabolic cell function and growth [16]. Decrease in cell proliferation suggests that metal ion release may indeed affect mitochondrial activity. Nevertheless, this hypothesis needs to be further confirmed.

It has been found that by affecting their proliferation, osteoblasts may delay the time course of their differentiation stages e.g. [17]. In the present study, we showed that metal ion release from the different surface treatments affected the cell proliferation. Therefore, it seems reasonable to assume that the different surface treatments may shift the time course of differentiation stage. A delay in the peak measurement of ALP activity was noted on the aged and the passivated samples compared to the control sample (Fig. 4). Even though results comparisons between two different cell lines should be handled carefully, it seems that the decrease of MG-63 proliferation and the delay in the peak measurement of SaOS-2 ALP activity agrees with the assumption mentioned that surface treatment could delay the differentiation pathway of the osteoblasts. A similar result has been demonstrated in a previous study [18].

The osteocalcin, osteopontin and osteonectin are important osteoblastic markers and are expressed at different maturation stages of the osteoblasts [19]. These three genes have been shown to be differently involved in the bone mineralization process. Osteocalcin, which is the most osteoblast-specific gene yet known [20], has been demonstrated to stimulate bone mineral maturation [21]. Osteopontin has been shown to inhibit the crystal growth of hydroxyapatite e.g. [22], while osteonectin has been suggested to promote collagen mineralization [23]. In the present study, no difference in

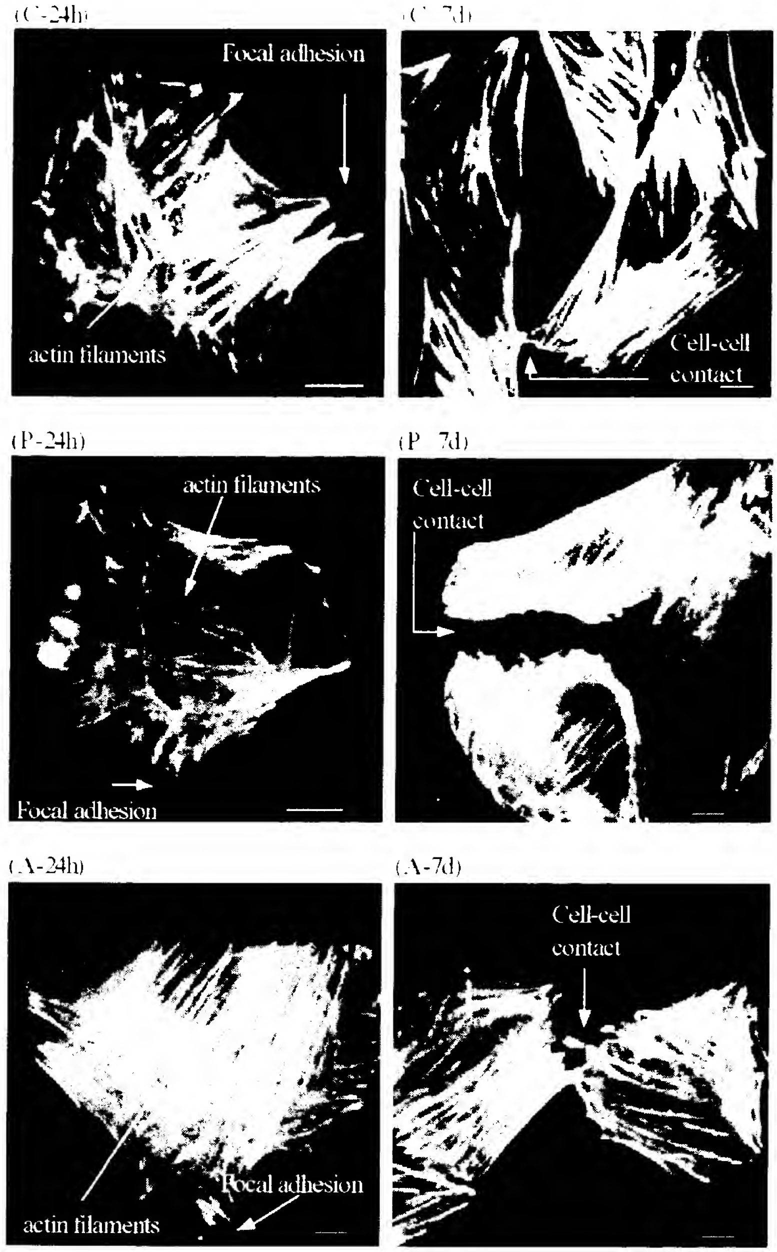

Fig. 6. Morphology of SaOS-2 cells seeded on the different Ti–6Al–4V samples at 24 h (C-24 h, P-24 h, A-24 h) and 7 days (C-7 d, P-7 d, A-7 d) (C: control, P: passivated; A: aged). Actin filaments and focal adhesion points were clearly visible at 24 h.

osteocalcin, osteopontin and osteonectin gene expression was found at week 1 between the samples. The different surface treatments seen then not to induce a different mineralization process, at least during the first week.

No FN was detected at week 1. Other proteins such as collagen may play a major role in the cell attachment to the different samples instead of FN at week 1 e.g. [24]. It may also be possible that the metal ion release from the samples affected the initial production of FN. A previous study has demonstrated that FN was not involved in the adhesion of osteoblasts to uncoated Ti alloy at 24 h [25]. Moreover, the integrin α_5 which is a major adhesion receptor of osteoblasts interacting with FN [26,27] was not detected in cell cultured on polished or rough Ti 6Al 4V at 12 h while it was detected for cell cultured on polystyrene [28]. Despite results of the study by Sinha and Tuan [28] were obtained at 12 h while present FN results were obtained at week 1, they could support the hypothesis that proteins other than FN were involved in the osteoblast adhesion to Ti alloy. The production of FN observed

after two weeks could then probably be due to cell–cell contact e.g. [29]. The increasing FN production at week 2 and 3 could also contribute to cell spreading configuration maintaining the survival of mature osteoblasts [30].

Previous work in our group has demonstrated that the different surface treatments alter the metal ion release kinetics and surface composition of the Ti 6Al 4V alloy [9,31]. The release of Al ions was found to be about 0.84 μM for the ageing treatment and about 5.55 μM for the passivation treatment after 7 days [9]. The osteoblasts cultured on the aged surface experienced therefore, a much lower concentration of Al ions during the test duration. The kinetics of the metal ion dissolution, especially for Al, could then explain the differences in cell behaviour, which were observed only in long-term in vitro study. Indeed, results of ALP peak activity at week 2 for the control treatment compared to the passivated and aged treatments was in agreement with a previous study using a similar Al ion concentration [32]. It should be noted also that the only difference in terms of ion release composition between the different Ti 6Al 4V surface treatments was the presence of vanadium for the control treatment. It may be possible therefore, that vanadium also has an impact on osteoblast differentiation by maturing the osteoblasts faster. Further experiments on this particular hypothesis need to be performed before a definitive conclusion can be drawn.

It has been shown that surface roughness influenced the cell behaviour [12]. We have used XPS and AFM techniques to examine the surface properties of the treated Ti 6Al 4V surfaces [33]. A difference in roughness (R_a) between the passivated and the aged samples could only be observed at a small scale (1 μm^2). The area average R_a was about 0.99 nm (C), 1.29 nm (P) and 0.56 nm (A). Therefore, at the cell level, the roughness could be considered as similar between samples and could not explain the differences in osteoblasts behaviour.

Biocompatibility tests such as those performed in this study can only quantify particular aspects of cell behaviour. The cell reaction to an implant is however a very complex situation and can only be partially understood using standard biological assays. In order to have a comprehensive description of the cell implant interaction with surface treated Ti alloy, cDNA microarray technology may be performed e.g. [34,35]. This represents the next step of this study.

5. Conclusions

In this study, short-term (24 h) in vitro experiments demonstrated that different Ti 6Al 4V surface treatments had negligible effects on the measured parameters. Cell viability remained unaffected and cell morphology expressed rich actin filaments. In longer in vitro experiments (from 72 h until 4 weeks), the difference in the kinetics of metal ion dissolution between treatments is more important and consequently a higher cell proliferation on the aged sample and an accelerated peak of ALP activity on the control sample were observed. Osteonectin, osteopontin, and osteocalcin gene expression (at week 1) as well as FN production (until week 4) were not affected by the different surface treatments. Based on our previous metal ion release studies and surface analyses, Al ions release kinetics as well as presence of vanadium ions may play a major role in influencing the osteoblasts behaviour in the present study.

Acknowledgements

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2-Methoxyestradiol Induces Interferon Gene Expression and Apoptosis in Osteosarcoma Cells

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2-Methoxyestradiol (2-ME), a naturally occurring mammalian metabolite of 17β-estradiol, has been implicated as a physiological inhibitor of tumor cell proliferation. In this study, the effects of 2-ME on cultured osteosarcomatous cells were investigated. Dose-dependent growth inhibition was observed in MG63 and TE85 human osteosarcoma cells exposed to 2-ME. The cell killing by 2-ME was ligand-specific; the immediate precursor (2-hydroxyestradiol), the parent compound (17β-estradiol), and the equivalent metabolite of estrone (2-methoxyestrone) exhibited less potency and efficacy. Furthermore, 2-ME was similarly effective at killing immortalized human fetal osteoblastic cells (hFOB) with and without estrogen receptor-α and -β and rat osteosarcoma cells (ROS17/2.8). The cytotoxicity of 2-ME was selective to transformed and immortalized osteoblastic cells; 2-ME (2 μm) had no effect on the proliferation of primary cultures of human osteoblasts. Co-treatment with the potent estrogen receptor ligand, ICI-182,780, did not reduce 2-ME-induced osteosarcoma cell death, implying that this action is not mediated by conventional estrogen receptors. The expression levels of bone matrix protein genes, type 1 collagen and osteonectin, were transiently reduced after 2-ME treatment, suggesting that the surviving cells are capable of producing bone matrix. The 2-ME-mediated killing of osteosarcoma cells was due to the induction of apoptosis; treatment induced expression of interferon genes within 12 h and histological evidence of apoptosis within 48 h of 2-ME treatment. Thus, our results demonstrate that 2-ME is highly cytotoxic to osteosarcoma cells but not normal osteoblasts. These findings suggest that further study of 2-ME as a potential intervention for treatment of osteosarcoma is warranted. (Bone 30: 393–398; 2002)

Key Words: Estrogen metabolism; Human bone cells; Osteoblasts; Osteosarcoma; Interferon; Apoptosis.

Introduction

Osteosarcoma is a malignant tumor of bone that is most prevalent in adolescents and young adults. Osteosarcoma accounts for approximately 5% of the tumors in childhood and 80% of these tumors originate around the knee.[7,15] The prognosis is often poor and, within 1 year after commencing definitive therapy, about 30% of patients diagnosed with osteosarcoma will develop lung metastasis.[20,26] The prognosis appears to be determined by the site of metastases and surgical resectability of the metastatic disease, either at diagnosis or following a variable period of chemotherapy. Patients who have complete surgical ablation of the primary and metastatic tumor (when confined to the lung) following chemotherapy may attain long-term survival, although event-free survival remains about 20% for patients with metastatic disease at diagnosis.[1,9] Patients developing recurrent disease often have a poor prognosis and die within 1 year of the development of metastatic disease. Chemotherapy is often ineffective, resulting in a high mortality rate. Hence, it is important that new therapeutic approaches are evaluated for this malignant disease.

2-Methoxyestradiol (2-ME) is an endogenous metabolite of 17β-estradiol, which is produced by sequential 2-hydroxylation and *O*-methylation.[8] 2-ME is present in human blood and urine and has been reported to inhibit endothelial cell proliferation and angiogenesis.[7,28,29] The mechanism of the antiangiogenic activity of 2-ME is not fully understood. Two potential mechanisms have been proposed: (1) disruption of the cytoskeleton during cell division; and (2) induction of apoptosis. 2-ME inhibits tubulin formation, causes disturbances in mitosis, and produces abnormal metaphase in some cell types.[6] In other cell types, 2-ME stimulates expression of the p53 gene, which also leads to the induction of apoptosis.[18,19]

The direct effects of 2-ME on osteoblasts have not been studied. However, administration of high doses of 2-ME to growing female rats did not disturb normal bone turnover.[24] In the present study, we evaluate the effects of 2-ME on survival of osteosarcoma cells in culture.

Materials and Methods

Cells and Culture Conditions

MG63, TE85 human osteosarcoma cells, and ROS17/2.8 rat osteosarcoma cells were grown in Dulbecco's modified eagle's medium (DMEM)/F12 medium containing 10% charcoal-stripped fetal bovine serum and supplemented with 100 units/mL penicillin and 100 μg/mL streptomycin. Human fetal osteoblast (hFOB) cells[10] and hFOB cells overexpressing estrogen receptor α (hFOB-ERα)[11] and estrogen receptor β (hFOB-ERβ)[27] were grown in DMEM/F12 medium containing fetal bovine serum (FBS), penicillin, streptomycin, and geneticin (300 μg/mL).

The laboratory of Dr. B. L. Riggs (Mayo Clinic) kindly

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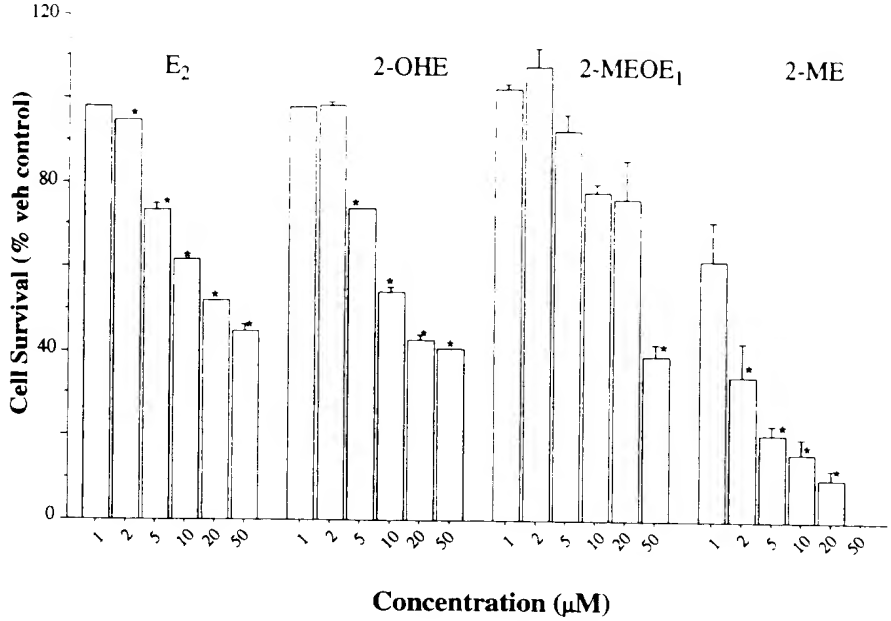

Figure 1. 2-Methoxyestradiol has greater potency and efficacy in killing MG63 osteosarcoma cells than either 17β-estradiol, 2-hydroxyestradiol, or 2-methoxyestrone. MG63 osteosarcoma cells were treated with vehicle, 0.1, 1, 2, 5, 10, 20, or 50 μmol/L 2-ME, 2-OHE, 2-MEOE$_1$, and E$_2$ for 72 h. The cells were harvested and the viable cell counts were taken after staining with trypan blue. Values are the mean ± SE (N = 3 replicate cultures). $^*p \leq 0.05$ (compared with vehicle, by one-way ANOVA and Fisher's PLSD analysis). From a semilogarithmic dose-response plot (not shown) we determined that 2-ME resulted in complete cell killing with an LD$_{50}$ of 1.5 μmol/L. The experiment shown is representative of 6–15 experiments. The absence of an error bar denotes a line thickness greater than error. Veh, ethanol vehicle control.

provided primary human osteoblast (hOB) cells. hOB cells were established from cancellous bone obtained as waste from orthopedic procedures in accordance with institutional regulations and cultured as explants to generate the osteoblast-like monolayers, as previously described.[3,21] hFOB cells and hFOB cells stably transfected with estrogen receptors (ERs) were maintained at 34°C and all other cells were incubated at 37°C under 5% CO_2 in air.

Metabolite Treatment and Cell Growth Assay

Cells were plated at 5×10^4 cells/well into 24 well plates containing 1 mL/well medium. After allowing the cells to attach overnight, the media in the wells were replaced with a fresh 1 mL of medium. ICI-182,780 was kindly provided by Zeneca Pharmaceuticals (Macclesfield, Cheshire, UK). The metabolites were added to each well containing 1 mL of medium and diluted 1000-fold to give the final required concentrations, and maintained for 72 h. 2-Methoxyestradiol (2-ME), 2-hydroxyestradiol (2-OHE), 2-methoxyestrone (2-MEOE$_1$), and 17β-estradiol (E$_2$) were purchased from Sigma Chemical Co. (St. Louis, MO) and the stock solutions at different concentrations were made in 95% ethanol.

Cell growth was measured by taking the viable cell count. At the end of metabolite treatment, cells were harvested, stained with trypan blue, and counted with the aid of light microscopy.

RNA Isolation

Cells were plated at 10^6 cells per flask in T-75 culture flasks 1 day prior to metabolite treatment. The next day, cells were replaced with fresh medium containing 10 μmol/L concentrations of 2-ME and incubated for different periods of time. The cells were harvested and the cell pellets were used for RNA isolation. Total cellular RNA was extracted and isolated using a modified organic solvent method and the RNA yields were determined spectrophotometrically at 260 nm.[5]

Northern Blot Hybridization

Total RNA samples (10 μg) were used for northern blot analysis as previously described.[16] Labeling of cDNAs, hybridization, and quantitation by PhosphorImager (Molecular Dynamics, Sunnyvale, CA) were carried out as previously described.[16] Labeled cDNAs for type I collagen, osteonectin, and 18S rRNA were used for probing the blots. The amounts of RNA loaded and transferred were assessed by methylene blue staining of the membranes and hybridization with a cDNA for 18S ribosomal RNA.

RNase Protection Assay for Cytokines

The total RNA isolated was analyzed by RNase protection assays using antisense RNA probes. The antisense RNA was synthesized using cDNA templates (Pharmingen, San Diego, CA). We measured the mRNA concentrations of the following cytokines: transforming growth factor-β (TGF-β)-1, 2, and 3; tumor necrosis factor-α and -β (TNF-α and -β); interleukin (IL)-1α, -1β, -1Ra, -6, -10, and -12 (p35 and p40); interferon (IFN)-β and -γ; and lymphotoxin (LT)-β. Quantitation of protected RNA fragments was performed by PhosphorImager analyses and normalized to glyceraldehyde 3-phosphate dehydrogenase and ribosomal structural protein L32.

Terminal Deoxynucleotidyl Transferase-mediated dUTP Nick End Labeling Assay

Apoptotic cells were detected using a terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) enzy-

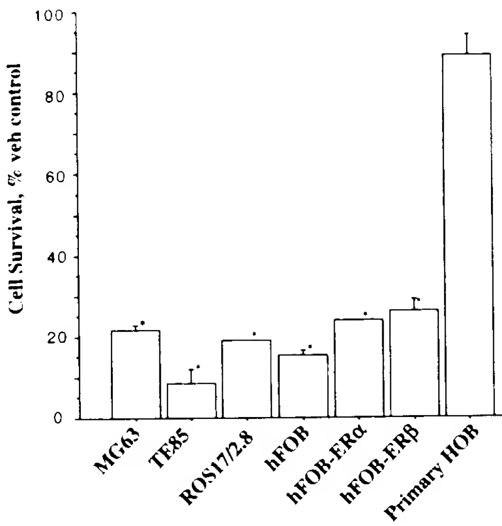

Figure 2. Effect of 2-ME on osteoblasts and osteoblast-like cells. Cells were treated with vehicle and 2 μmol/L 2-ME for 72 h. The cells were harvested and the viable cell counts were taken after staining with trypan blue. Values are the mean ± SE (N = 3 replicate cultures). *$p \leq 0.05$ (compared to vehicle using one-way ANOVA and Fisher's PLSD analysis). Dose-response studies revealed (data not shown) that the LD_{50} values for TE85 and ROS17/2.8 cells were approximately 4 and 1 μmol/L, respectively. The experiment shown is representative of three experiments. The absence of an error bar denotes a line thickness greater than the error. Veh, ethanol vehicle control.

matic labeling assay as per the manufacturer's protocol (Boehringer Mannheim, Indianapolis, IN), which directly labels the ends of broken DNA strands with fluorescein-dUTP. Apoptotic cells in adherent cultures were detected by fluorescence microscopy. In brief, the TUNEL assay was performed on coverslips of adherent cells contained in individual wells of a 24 well culture dish. Cells were washed once with phosphate-buffered saline (PBS) and allowed to air dry before being fixed in 4% paraformaldehyde prepared in PBS (Sigma) for 1 h at room temperature. Following a PBS rinse, endogenous peroxidase was blocked by incubating cells for 1 h at room temperature with 0.3% hydrogen peroxide in methanol (Sigma). The latter was performed for the option of converting fluorescein-labeled cells to peroxidase-labeled cells and DAB chromogenic detection. After the PBS rinse, cells were permeabilized on ice with incubation in 0.1% Triton X-100 in 0.1% sodium citrate for 2 min. Coverslips were rinsed twice in PBS before the addition of TUNEL reaction mixture (100 μL). One well containing untreated cells was treated with 2 μg/mL DNase I (Promega, Madison, WI) for 10 min at room temperature to serve as a positive control for the TUNEL reaction. Coverslips were incubated in TUNEL reaction mixture for 1 h at 37°C. Coverslips were rinsed three times with PBS before visualization by fluorescence microscopy. Negative controls consisted of three different treatments: (1) 2-ME-treated cells incubated 1 h at 37°C in the absence of transferase enzyme in the TUNEL mixture; (2) cells treated with vehicle only; and (3) untreated adherent cells.

Statistical Analysis

All values are expressed as means ± standard error. Significant differences between groups were determined by Fisher's protected least significant difference post hoc test for multiple-group comparisons following detection of significance by one-way analysis of variance (ANOVA). $p < 0.05$ was considered statistically significant.

Results

Effect of Estrogen and Estrogen Metabolites on Survival of Osteosarcomatous and Osteoblastic Cells

The growth of MG63 osteosarcoma cells was examined in the presence of estrogen metabolites 2-ME, 2-OHE, 2-MEOE$_1$, and

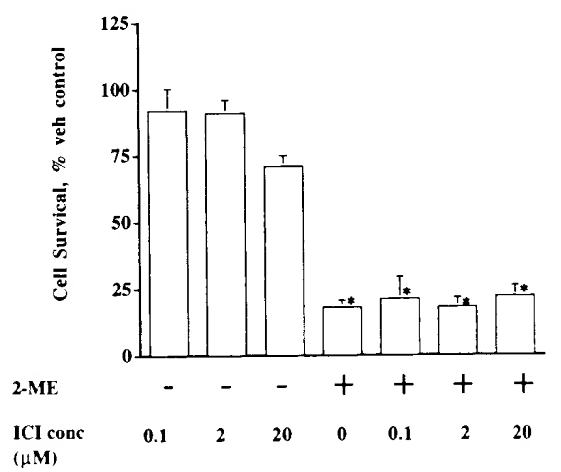

Figure 3. Antiestrogen ICI-182,780 does not block the 2-ME induced killing of osteosarcoma cells. MG63 Cells were treated with vehicle 2 μmol/L 2-ME in the presence and absence of 0.1, 2, and 20 μmol/L concentrations of ICI-182,780 (ICI) for 72 h. The cells were harvested and the viable cell counts were taken after staining with trypan blue. Values are the mean ± SE (N = 3 replicate cultures). *$p \leq 0.05$ (compared with vehicle using one-way ANOVA and Fisher's PLSD analysis). Results are representative of three independent treatments. Veh, ethanol vehicle control.

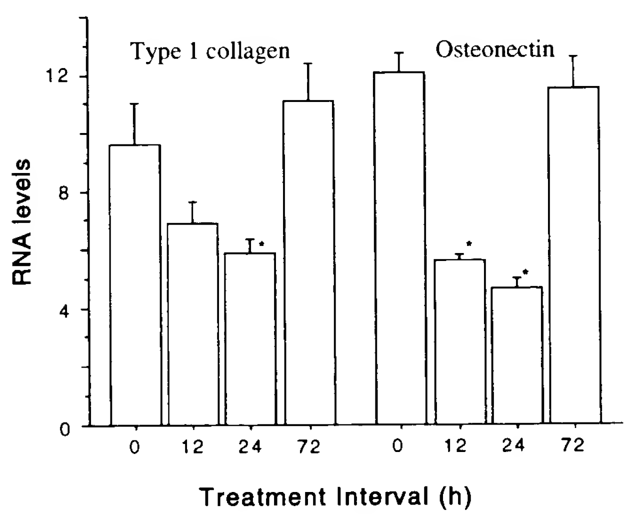

Figure 4. 2-ME treatment leads to a decrease in bone matrix gene expression. MG63 cells in triplicate cultures were treated with 10 μmol/L 2-ME. Cells were harvested at the end of 0, 12, 24, and 72 h of treatment and used for RNA isolation. Total RNA isolated from cells was analyzed by northern blot hybridization. Values are the mean ± SE (N = 3 replicate cultures). *$p \leq 0.05$ (compared with time point 0 using one-way ANOVA and Fisher's PLSD analysis).

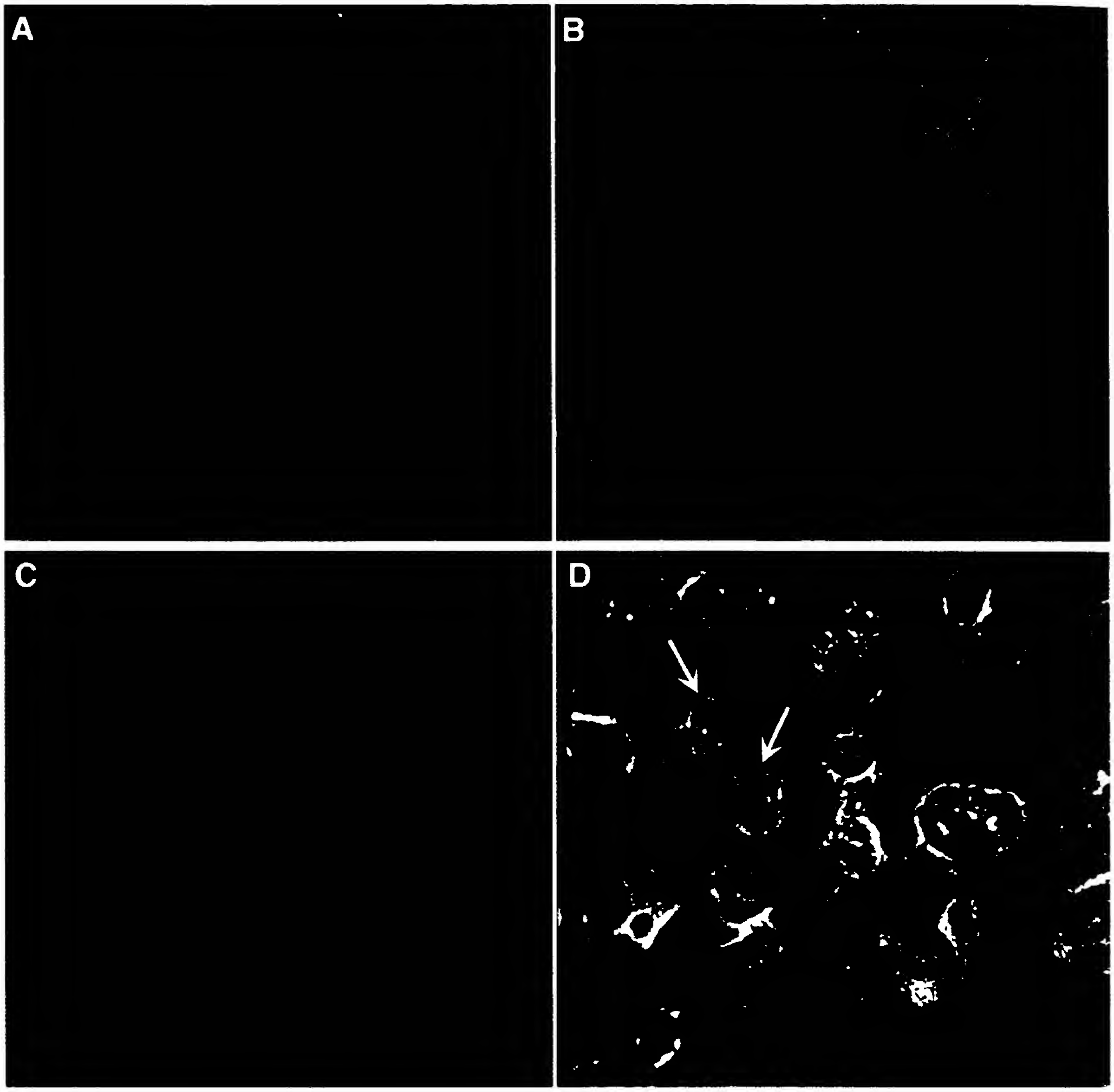

Figure 5. Detection of apoptosis by TUNEL assay in 2-ME-treated MG63 cells. Cells were cultured as described in legend to Figure 1 in the presence and absence of 2-ME. (A) Veh, ethanol vehicle control showing no stain. (B) Positive control where DNA breaks were introduced by DNase I treatment. (C) UV, cells treated with 10 μmol/L 2-ME and analyzed under ultraviolet light. (D) VIS + UV, cells treated with 10 μmol/L 2-ME and analyzed under visible and ultraviolet light.

the parent compound E_2 (**Figure 1**). Viable cell counts taken after 72 h of treatment revealed that about 80% of the cells were killed at 2 μmol/L of 2-ME alone. At 20 and 50 μmol/L, about 95% and 100% cell death was observed with 2-ME. On the other hand, the 2-OHE, 2-MEOE$_1$, and E_2 treatments resulted in partial killing.

When the effects of 2 μmol/L 2-ME on the proliferation of several osteoblastic cell lines at the end of 72 h of treatment were investigated, we observed that the survival rates for human osteosarcoma cells (MG63 and TE85) and rat osteosarcoma cells (ROS17/2.8) were reduced to 21%, 9%, and 19%, respectively (**Figure 2**). The cell numbers for immortalized human FOB cells, FOB cells expressing ERα, and FOB cells expressing ERβ were also reduced to 15%, 24%, and 24%, respectively. However, the survival rate for normal hOB cells derived from adult patients was 89%.

Effect of Antiestrogen ICI-182,780 on 2-ME-mediated Cell Killing

To determine whether the 2-ME-mediated cell killing in osteosarcoma requires binding to either ERα or ERβ, the cytotoxic effect of 2-ME on MG63 was followed in the presence and absence of ICI-182,780 (**Figure 3**). 2-ME at 2 μmol/L decreased MG63 cell survival to <20% compared with vehicle controls. Cotreatment of ICI-182,780 at the 0.1, 2 (1×), and 20 (10×) μmol/L concentrations of 2-ME used in this experiment did not block cell killing by 2-ME. Also, ICI-182,780 had no indepen-

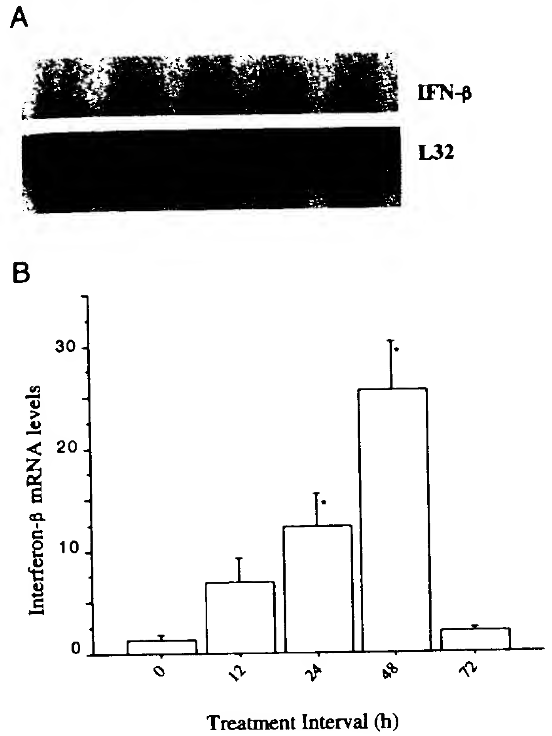

Figure 6. 2-ME induces interferon-β mRNA levels. (A) Gel. (B) Quantitation by PhosphorImager. MG63 cells in triplicate cultures were treated with 10 μmol/L 2-ME. Cells were harvested at the end of 0, 12, 24, 48, and 72 h of treatment and used for RNA isolation. Total RNA isolated from the cells was analyzed by RNase protection assay using the human cytokine template set for multiprobes (BD Pharmingen, San Diego, CA). Quantitation of protected RNA fragments was performed by PhosphorImager analyses and normalized to L32 mRNA levels. Values are expressed as mean ± SE (N = 3 replicate cultures). $*p \leq 0.05$ (compared with timepoint 0, using one way ANOVA and Fisher's PLSD analysis).

dent effect on cell survival at 0.1 and 2 μmol/L (lower) concentrations. Very high concentrations of ICI-182,780 (20 μmol/L) slightly reduced cell survival to 71%.

Effect of 2-ME on Bone Matrix Gene Expression

The time-course effects of 2-ME (10 μmol/L) on steady-state mRNA levels for bone matrix proteins were determined (**Figure 4**). 2-ME treatment led to transient decreases in type 1 collagen mRNA levels by 26% and 37% and osteonectin by 50% and 38% after 12 and 24 h, respectively. The mRNA levels for the bone matrix proteins returned to normal levels by 72 h.

Induction of Programmed Cell Death by 2-ME in Osteosarcoma Cells

We examined the effect of 2-ME on in situ apoptosis at the single cell level, based on labeling of DNA strand breaks using the TUNEL method. **Figure 5**C shows typical positive staining of MG63 osteosarcoma cells after exposure to 10 μmol/L of 2-ME for 48 h. In contrast, cells exposed to vehicle for 48 h had no staining (Figure 5A). In an untreated control, where the DNA breaks were introduced by DNase I treatment, there was positive staining (Figure 5B). Untreated MG63 control and cells exposed to 2-ME but incubated in the absence of transferase enzyme in the TUNEL mixture showed no staining (not shown).

Effect of 2-ME on Cytokines and Growth Factors

We measured the changes in steady-state mRNA levels for cytokines that have been implicated in the regulation of bone formation and resorption. We analyzed the mRNA concentrations for members of the interleukin (IL) family, IFNs TNF, and TGF-β by RNase protection assay. Although many cytokine genes, including IL-1α, IL-1β, IL-1Ra, IL-6, IL-10, IL-12, IFN-γ, TNF-α, TNF-β, and lymphotoxin-β were not detected in either control or 2-ME-treated cultures, there were 2-ME-induced increases in the mRNA levels of IFN-β, TGF-β1, TGF-β2, and TGF-β3. The IFN-β mRNA level was increased by 500% within 12 h of 2-ME treatment and reached a maximum of 2100% by 48 h (**Figure 6**). TGF-β1, TGF-β2, and TGF-β3 mRNA levels were increased by 177%, 289%, and 178%, respectively (data not shown), and thereby showed a modest response to 2-ME treatment.

Discussion

2-ME treatment resulted in the inhibition of osteosarcoma cell growth, at least in part by decreasing cell survival. This dose-dependent cell death was dramatic and specific to osteosarcoma and immortalized osteoblastic cell lines. The growth of normal human osteoblasts was not affected, suggesting that 2-ME may be useful as a therapeutic agent for bone metastasis. The lack of toxic effects of high dose rates of 2-ME on normal osteoblasts has also been confirmed in rats.[24] Although 2-ME-mediated growth inhibition and cell death have been documented in various tumor cells,[19,22,23,28] this is the first report of 2-ME inducing apoptotic cell death in a cancer cell of bone origin. The induction of cell death is found to be specific to 2-ME, as its immediate precursor, 2-OHE, showed reduced potency and efficacy. 2-OHE, 2-MEOE$_1$, and E$_2$ were also cytotoxic to osteosarcoma cells, albeit to a lesser extent than 2-ME. 2-OHE, 2-MEOE$_1$, and E$_2$ may have inherent cytotoxic activity or, alternatively, their activities may be due to further metabolism to 2-ME. 2-Hydroxylase and methyltransferase have been shown to be widely distributed.[2] Further studies are necessary to determine whether these two enzymes are expressed in osteoblasts.

E$_2$, which has a 2000-fold higher binding affinity to the estrogen receptor than 2-ME,[17] has much less effect than its metabolite on osteosarcoma cell survival. This finding suggests that the toxic effects of 2-ME are not mediated through conventional estrogen receptors, a conclusion supported by two additional independent lines of evidence. First, 2-ME was equally effective in killing cells expressing both low and high levels of endogenous estrogen receptors. Second, the cytotoxic effects of 2-ME were neither antagonized nor potentiated by the high-affinity estrogen receptor ligand, ICI-182,780.

The positive TUNEL assay indicated that 2-ME induced cell death in osteosarcoma cells by apoptosis. The observed transient reductions in expression of type 1 collagen suggests that the estrogen metabolite inhibits osteoblast activity, because a strong correlation between mRNA levels for this bone matrix protein and histomorphometric indices of bone formation have been shown.[25] The parallel change in mRNA levels for osteonectin supports this conclusion.

The IFN-β- and TGF-β-mediated induction of apoptotic pathways has been well established.[4,12–14] However, this is the first study implicating these cytokines in 2-ME-induced programmed cell death. Earlier studies have implicated a p53-gene-mediated pathway in 2-ME induction of programmed cell death.[18,19] Thus,

2-ME may induce multiple pathways leading to programmed cell death.

Although the physiological significance of 2-ME is not yet clear, this naturally produced estrogen metabolite has highly desirable properties that clearly discriminate between normal and tumor cell growth.[23] Further investigation of the therapeutic potential of 2-ME for treatment of osteosarcoma is warranted.

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SPARC Is a Key Schwannian-derived Inhibitor Controlling Neuroblastoma Tumor Angiogenesis[1]

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ABSTRACT

Neuroblastoma (NB), a common pediatric neoplasm, consists of two main cell populations: neuroblastic/ganglionic cells and Schwann cells. NB tumors with abundant Schwannian stroma display a more benign clinical behavior than stroma-poor tumors. Recent studies suggest that Schwann cells influence NB tumor growth via secreted factors that induce differentiation, suppress proliferation, and inhibit angiogenesis. Two angiogenesis inhibitors, pigment epithelium-derived factor and tissue inhibitor of metalloproteinase-2, have been detected in Schwann cell secretions. Here, we isolated another Schwann cell-derived secreted inhibitor of angiogenesis, a 43-kDa protein identified as SPARC (secreted protein acidic and rich in cysteine), an extracellular matrix protein. We found SPARC to be critical for the antiangiogenic phenotype of cultured Schwann cells. We also show that purified SPARC potently inhibits angiogenesis and significantly impairs NB tumor growth *in vivo*. SPARC may be an effective candidate for the treatment of children with clinically aggressive, Schwannian stroma-poor NB tumors.

INTRODUCTION

NB,[3] a common pediatric neoplasm that arises from neural crest tissue, has a broad spectrum of clinical behavior (1–4). Although numerous factors including stage (5), patient age (6), tumor histology (7), molecular markers (8, 9), and genetic abnormalities (10–13) have been shown to be predictive of outcome in children with NB, the mechanisms responsible for the highly variable clinical behavior of NB remain largely unknown. Several recent studies implicate angiogenesis in the regulation of NB growth. In primary NB tumors, high vascular index correlates with *MYCN* amplification, metastases, and poor outcome, whereas low tumor vascularity is associated with a better prognosis, localized stage, and favorable histology (14). Advanced-stage NB is associated with high levels of angiogenic stimuli and $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins, both markers of active angiogenesis (15, 16). Overexpression of exogenous *MYCN* results in enhanced malignant growth of NB cells and reduced levels of activin A, an inhibitor of angiogenesis (17). Expression of the neurotrophin receptor TrkA also causes down-regulation of angiogenesis stimulators and impaired tumorigenicity in a mouse xenograft model (18). Furthermore, preclinical studies have shown effective reduction of NB tumor growth *in vivo* by a variety of antiangiogenesis agents (16, 19–23).

NB tumors consist of two main cell populations, neuroblastic/ganglionic cells and Schwann cells (24). The ratio of these cell types varies according to tumor maturation. Immature NB tumors are composed of undifferentiated neuronal cells and a paucity of Schwannian stroma, whereas larger, ganglion-like cells and abundant Schwannian stroma are seen in maturing NB tumors. The Schwann cells are thought to be normal cells that infiltrate the NB tumor (25), although a recent report suggests that the Schwann cells may be malignant (26). The favorable prognostic impact of the presence of Schwannian stroma has been emphasized in the pathological classification system of Shimada *et al.* (24) and the International NB Pathology Classification System (7). It has been speculated that Schwann cells influence NB tumor growth by secreting molecules that serve as antiproliferative and differentiating factors for neuronal cells (25, 27, 28). Schwann cells also produce a spectrum of angiogenesis inhibitors (29, 30), suggesting that Schwann cells may also influence NB growth by restricting angiogenesis.

The angiogenesis inhibitors PEDF and TIMP-2 have been previously found in media conditioned by Schwann cells, and both factors appear to contribute to the antiangiogenic activity of the SCM (29, 30). In this study, we isolated an additional angiogenic inhibitor in SCM, identified as SPARC. SPARC, also known as osteonectin, BM-40, and 43K protein, is a highly conserved calcium-binding matricellular glycoprotein (31–33). SPARC is spatially and temporally regulated during development, and it is transiently expressed in derivatives of the three primitive germ layers in mouse embryos (34). This glycoprotein is highly expressed in bone and in basement membranes as well as in a variety of cell types associated with remodeling tissues and high cellular turnover (35). Although its precise function is unclear, SPARC plays a modulatory role in cell-matrix interactions (36). SPARC induces cell rounding, blocks cell spreading and adhesion, and inhibits endothelial cell migration (37–39). SPARC also appears to contribute to vascular morphogenesis and cellular differentiation (36). However, the contradictory reports regarding the role of SPARC in cell growth and tumor formation (40–42) suggest that its effects are cell type specific and may be dependent on concentration, extracellular matrix components, and the ability of the cell to proteolyze SPARC (42–44).

We report that SPARC expression is inversely correlated with the degree of malignant progression in NB tumors. We also demonstrate that SPARC is one of the key contributors to the antiangiogenesis activity of the SCM. Purified SPARC blocks angiogenesis *in vitro* and *in vivo* and significantly impairs NB tumor growth *in vivo*. Our observations stress the cumulative nature of the angiogenic equilibrium in NB tumors and suggest that a shift in the angiogenic balance may be prompted by even slight disturbances in the complex array of inhibitors and stimuli in the endothelial cell microenvironment.

MATERIALS AND METHODS

Cell Culture and CM Collection. Primary human Schwann cells were purified from adult nerves or from Schwannian stroma-dominant NB tumors and expanded as described previously (29, 30, 45). Tumor-derived Schwann

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[3] The abbreviations used are: NB, neuroblastoma; PEDF, pigment epithelium-derived factor; TIMP-2, tissue inhibitor of metalloproteinase-2; SCM, Schwann cell-conditioned media; RA, retinoic acid; BrdUrd, 5-bromo-2′-deoxyuridine; CM, conditioned media; MVD, microvascular density; bFGF, basic fibroblast growth factor; RT-PCR, reverse transcription-PCR.

cells were maintained at 5% CO_2 in DMEM (Life Technologies, Inc., Grand Island, NY) supplemented with 10% fetal bovine serum (US Biotechnologies, Inc., Parkerford, PA), 50 ng/ml recombinant human heregulin $\beta1$ (R&D Systems, Minneapolis, MN), 1% penicillin/streptomycin, 2.5 μg/ml amphotericin, 0.5 μM isobutylmethylxanthine (Sigma, St. Louis, MO), and 0.5 μM forskolin (Sigma). NB cell lines used in this study have been described previously (46–49), with the exception of NBL-L and NBL-R, *MYCN*-amplified lines established in our laboratory from clinically aggressive NB tumors. NB cells were grown at 5% CO_2 in RPMI 1640 (Life Technologies, Inc.) supplemented with 10% fetal bovine serum and antibiotics. For some experiments, 10 μM all-*trans*-RA (Sigma) or 6 μM BrdUrd (Sigma) was added where indicated, and the cells were harvested at the indicated time intervals. CM from NB cell lines and SCM were collected as described previously (29, 30).

Isolation of SPARC from SCM. Concentrated SCM were dialyzed against PBS and fractionated on a HiTrap Q-Sepharose column (Amersham, Piscataway, NJ) with a 0.1–1.0 M NaCl gradient in 20 mM Tris-HCl (pH 8.0). Fractions that blocked endothelial cell chemotaxis or induced endothelial cell apoptosis were dialyzed against loading buffer, fractionated using a HiTrap heparin-Sepharose column (Amersham; 0.1–1.0 M NaCl gradient), and subjected to identical functional assays. Fractions were analyzed by SDS-PAGE followed by silver staining. A common 43-kDa band in the inhibitory flow-through fractions was cut from a Coomassie Blue-stained gel and submitted for sequence analysis at the Harvard University Microchemistry Facility.

SPARC Expression Analysis. To examine SPARC mRNA expression levels, total RNA was isolated from cultured cells using Trizol reagent (Life Technologies, Inc.) according to the manufacturer's instructions, and 1 μg of total RNA was reversed transcribed using Superscript II (Life Technologies, Inc.). To detect the SPARC transcripts, semiquantitative RT-PCR was performed using template diluted 1:100 and the sense primer 5′-CTGCCTGCCACTGAGGGTTCC-3′ and antisense primer 5′-TCCAGGCAGAACAACAAACCATCC-3′. β-Actin was used as a loading control with template diluted 1:1000 and sense primer 5′-TGTTGGCGTACAGGTCTTTGC-3′ and antisense primer 5′-GCTACGAGCTGCCTGACGG-3′. All targets were amplified for 30 cycles at an annealing temperature of 60°C. SPARC mRNA levels were also analyzed using real-time RT-PCR as described previously (50). For the quantitative real-time RT-PCR experiments, the primer pair 5′-TCTTCCCTGTACACTGGCAGTTC-3′ (sense) and 5′-AGCTCGGTGTGGGAGAGGTA-3′ (antisense) was used with the probe Fam-CAGCTGGACCAGCACCCCATTGA-QSY7. SPARC protein levels in CM were examined by Western blots. Briefly, CM were concentrated 50-fold using 5K cutoff centrifugal filter devices (Millipore, Bedford, MA). Total protein (10 μg) was electrophoresed in a 4–20% SDS-PAGE gradient gel and transferred to a nitrocellulose membrane (Bio-Rad, Richmond, VA) using standard techniques (51). After transfer, the blots were stained with Ponceau S (Sigma) to confirm equal loading. Membranes were blocked with 5% nonfat dry milk for 1 h and then incubated for 2 h with anti-osteonectin antibody (referred to hereafter as anti-SPARC antibody; Zymed, San Francisco, CA) at a 1:2000 dilution. The membranes were washed three times with PBT (PBS with 0.1% Tween 20) and then incubated for 2 h with a 1:2000 dilution of horseradish peroxidase-conjugated secondary antibody (Kirkegaard and Perry Laboratories, Gaithersburg, MD). The bound antibody complexes were detected using the LumiGLO chemiluminescence reagent (KPL).

Immunohistochemistry Studies. Histological sections of human NB and ganglioneuromas (mature Schwannian stroma-rich NB) were immunostained using a mouse anti-SPARC monoclonal antibody (Zymed). Briefly, paraffin-embedded NB tumor tissue fixed in 10% buffered formalin was sliced into 4-μm-thick sections, rehydrated in graded alcohols, and rinsed in PBS. Antigen retrieval was performed with 0.01 M citrate buffer (pH 6.0) in a boiling steamer (20 min). Sections were incubated overnight with primary antibody (1:1600 dilution) at 4°C and developed with peroxidase labeled-dextran polymer followed by diaminobenzidine (Envision Plus System; DAKO Corp., Carpinteria, CA). Sections were counterstained with Gill's hematoxylin. A human Schwannoma sample was included in each assay as a positive control, and staining without primary antibody was used as a negative control. Cytoplasmic patches of brown color were scored as SPARC positive. Rat antimouse monoclonal CD31 (PECAM-1) antibody (1:100 dilution, Research Diagnostics Inc., Flanders, NJ) was used to highlight endothelial cells on frozen tumor sections. MVD was quantified by counting 10 consecutive fields at ×200 magnification, and the average MVD counted in the 10 fields was reported as MVD/mm^2.

***In Vitro* Angiogenic Assay.** Migration assays were performed with human umbilical vein endothelial cells [National Cancer Institute Preclinical Repository (Bethesda, MD) and VEC Technologies (Rensselaer, NY)] as described previously (29). Test substances were assayed in Opti-MEM media (Life Technologies, Inc.) with or without 3 ng/ml bFGF (National Cancer Institute Preclinical Repository). Purified human platelet osteonectin (referred to hereafter as SPARC) was obtained from Calbiochem (San Diego, CA). To generate dose-response curves, the data were normalized as percentage of maximum migration using the difference between bFGF/Opti-MEM-induced migration and background migration in Opti-MEM alone as 100% control. For some assays, neutralizing anti-SPARC antibody (Zymed) or isotype-matched control antibody was added to the media at 10 μg/ml after dialysis against PBS. Control mouse IgG was obtained from Lab Vision (Fremont, CA).

Endothelial Cell Apoptosis Assay. Bovine adrenal capillary endothelial cells were treated overnight with SCM or control Opti-MEM. Dialyzed anti-SPARC antibody (Zymed) and control antibody were added at 10 μg/ml in some assays. Apoptotic cells were visualized using the ApopTag *in situ* Apoptosis Detection kit (InterGen, Gaithersburg, MD). Each assay was performed in triplicate, and the percentage of apoptotic cells was calculated as the number of green terminal deoxynucleotidyl transferase-mediated nick end labeling-positive cells with DNA fragmentation divided by the total number of Hoechst-counterstained nuclei.

***In Vivo* Angiogenesis Assay.** Female Fischer 344 rats (Harlan, Madison, WI) were used to perform rat corneal assays using previously described methods (29, 52). Briefly, 5 μl Hydron pellets (IFN Sciences, New Brunswick, NJ) prepared with 25 μg/ml SPARC (Calbiochem) with or without 50 ng/ml bFGF were implanted into the corneas of anesthetized rats. Control studies were performed with pellets containing PBS with or without bFGF. Additional experiments were performed with pellets also containing 50 μg/ml anti-SPARC antibody (Zymed). After 7 days, the animals were sacrificed and perfused with waterproof drawing ink (Sanford, Bellwood, IL) by intracardiac injection. The eyes were fixed in 10% neutralized buffered formalin overnight. The corneas were examined, and dense capillaries reaching the pellet were scored as positives. Animals were treated according to NIH guidelines for animal care and use, and protocols were approved by the Animal Care and Use Committee at Northwestern University.

***In Vivo* Inhibition of NB Growth.** NB xenografts were grown in female 4–6-week-old homozygous athymic nude mice (Harlan) after s.c. inoculation of 5×10^6 SMS-KCNR NB cells into the right flank. Once tumors were palpable, animals were anesthetized, and ALZET osmotic pumps (Durect, Cupertino, CA) containing purified SPARC (Calbiochem; $n = 3$) or PBS ($n = 3$) were implanted s.c. SPARC was released s.c. by the pump at a rate of 62.5 ng/h. Tumor volume was measured weekly using the formula: tumor volume = (length × width^2)/2 (53). Mice were sacrificed after 3 weeks of treatment, and tumors were resected for histological analysis. Tumor volume was analyzed using Student's t test to compare control and treatment groups.

RESULTS

Schwann Cells Secrete Antiangiogenic SPARC. We have previously reported antiangiogenic activity in SCM collected from normal and NB tumor-derived Schwann cells (29) and demonstrated the presence of several angiogenic inhibitors including PEDF and TIMP-2 (29, 30). Seeking additional angiogenesis inhibitors in SCM, we subjected it to multiple-step chromatography and tested fractions for the ability to block bFGF-induced endothelial cell migration and cause endothelial cell apoptosis. Fractions capable of both activities contained a 43-kDa protein (Fig. 1) that was identified as SPARC using sequence analysis of the tryptic peptide fragments. No contaminating sequence was detected in the SPARC band.

SPARC Was Expressed by Schwann Cells and Differentiated NB Cells *in Vitro* and *in Vivo*. SPARC expression was evaluated by semiquantitative RT-PCR in Schwann cells, a panel of NB cell lines, and in phenotypically distinct subclones of NB cell lines [neuroblastic (N-type) and substrate adherent (S-type)] that exhibit different malig-

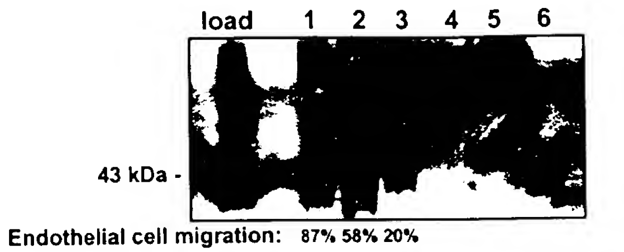


Fig. 1. Silver-stained gel of SCM fractions collected after heparin-Sepharose chromatography. The 43-kDa protein detected in *Lane 3* was identified as SPARC. Fraction 3 potently inhibited bFGF-induced endothelial cell migration as indicated at the *bottom* of the gel.

nant potentials (Refs. 54 and 55; Fig. 2*A*). Although SPARC mRNA was detected in all NB cell lines with the exception of NBL-W-N, mRNA levels were significantly higher in the Schwann cells and nontumorigenic S-type subclones than in tumorigenic N-type subclones and NB cell lines. SPARC protein levels in CM collected from the cells paralleled the mRNA levels (Fig. 2*B*). NB cells can be induced to differentiate *in vitro* with a number of agents including all-*trans*-RA or BrdUrd (56–59), and real-time quantitative RT-PCR demonstrated up to a 10-fold increase in SPARC mRNA in differentiated NB cells (Fig. 2*C*).

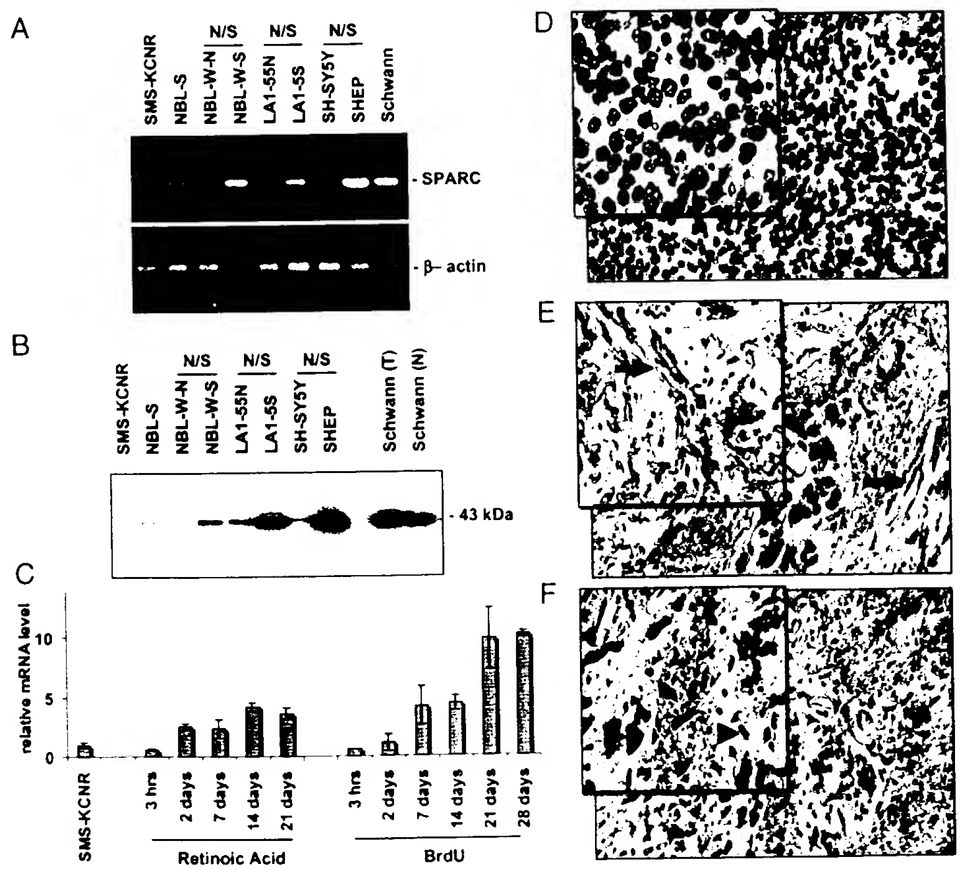
To investigate whether SPARC was expressed within NB tumors, histological sections from NB tumors displaying varying degrees of differentiation and abundance of Schwannian stroma and from ganglioneuromas were stained with antibody against human SPARC. Schwannian stroma-poor tumors were composed predominantly of neuroblasts and showed minimal or no staining for SPARC (Fig. 2*D*). Conversely, in maturing and mature tumors, SPARC could be detected not only in Schwann cells (Fig. 2*E*) but also in differentiating neuroblasts/ganglion cells (Fig. 2*F*).

SPARC in SCM Inhibited Migration and Induced Apoptosis of Endothelial Cells. The previously reported inhibition of bFGF-induced endothelial cell migration by SCM (29) was SPARC dependent because migration was largely restored in the presence of neutralizing antibody against SPARC (Fig. 3*A*). We also showed that SPARC-dependent induction of endothelial cell apoptosis by SCM was effectively neutralized by the same antibody (Fig. 3*B*). Consistent with earlier studies (38), purified SPARC blocked bFGF-induced endothelial cell migration in a dose-dependent manner at concentrations ranging from 0.05 to 5 μg/ml (Fig. 3*C*). However, endothelial cell migration inhibition was not observed at higher concentrations of SPARC. Biphasic responses have similarly been reported with the angiogenesis inhibitor thrombospondin-1 (60, 61). SPARC also triggered endothelial cell apoptosis, with maximal induction at 20 μg/ml (Fig. 3*D*).

SPARC Inhibited Angiogenesis and Impaired Tumor Growth *in Vivo*. Purified SPARC blocked bFGF-induced angiogenesis *in vivo* in the rat corneal neovascularization assay (Fig. 4; Table 1). Furthermore, the addition of the anti-SPARC antibody fully restored angiogenesis by bFGF, indicating that this inhibitory effect was indeed due to SPARC. Angiogenesis was not observed when SPARC was tested alone (Fig. 4). To our knowledge, the ability of SPARC to inhibit angiogenesis has not previously been tested in a rat corneal model.

The effect of SPARC on NB growth *in vivo* was tested in a mouse xenograft model where SPARC was delivered continuously for 3 weeks using osmotic pumps. During the first 2 weeks, tumor growth was completely arrested in the SPARC-treated group, whereas in the control animals carrying PBS-charged pumps, the volume of the

Fig. 2. SPARC expression in NB and Schwann cells. *A*, SPARC mRNA was measured in NB cell lines and Schwann cells by semiquantitative RT-PCR. Higher levels of expression were observed in nontumorigenic S-type NB subclones and Schwann cells than in tumorigenic NB cell lines and N-type subclones. *B*, SPARC protein was measured in media conditioned by NB cell lines, normal Schwann cells (*N*), and tumor-derived Schwann cells (*T*) by Western blot analysis. Higher levels of secreted protein were detected in the nontumorigenic S-type subclones and Schwann cells compared with tumorigenic NB cell lines. *C*, SPARC expression, measured by real-time quantitative RT-PCR, was up-regulated in SMS-KCNR NB cells induced to differentiate with RA or BrdUrd. *D*, SPARC is not detected by immunoperoxidase staining in an undifferentiated, Schwannian stroma-poor NB (magnification, ×400; *inset*, ×600). Immunoperoxidase staining of a ganglioneuroma (Schwannian stroma-dominant tumor; *E*) and differentiating NB (*F*) demonstrates SPARC expression in Schwann cells (*arrows*) and differentiating neuroblasts/ganglion cells [*arrowheads*; (magnification, ×200; *insets*, ×600)].



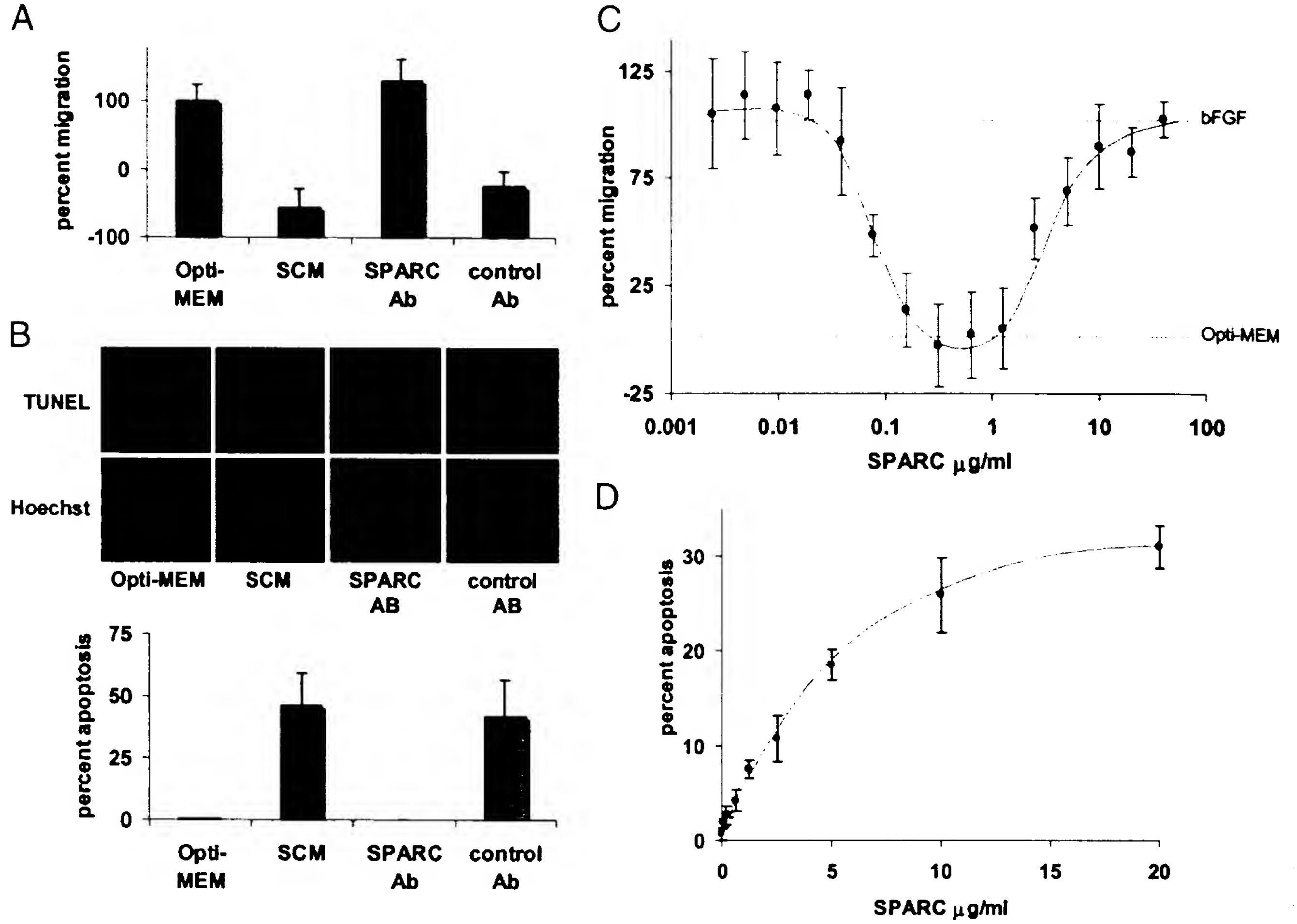


Fig. 3. SPARC inhibits angiogenesis *in vitro*. *A*, neutralizing anti-SPARC antibody reverses SCM inhibition of bFGF-induced endothelial cell migration. No effect is seen with control antibody. bFGF-induced endothelial cell migration is observed in the Opti-MEM control. *B*, terminal deoxynucleotidyl transferase-mediated nick end labeling-positive cells (*top panels*) with Hoechst-counterstained nuclei (*bottom panels*). Protection of endothelial cells from SCM-induced apoptosis was observed in the presence of anti-SPARC neutralizing antibody but not control antibody. The percentage of endothelial cell apoptosis for each experiment is shown in the *bar graph*. *C*, purified SPARC inhibits bFGF-induced endothelial cell migration with an ED_{50} of ~2 nM. *D*, purified SPARC triggers endothelial cell apoptosis in a dose-dependent manner with maximal response at 20 μg/ml.

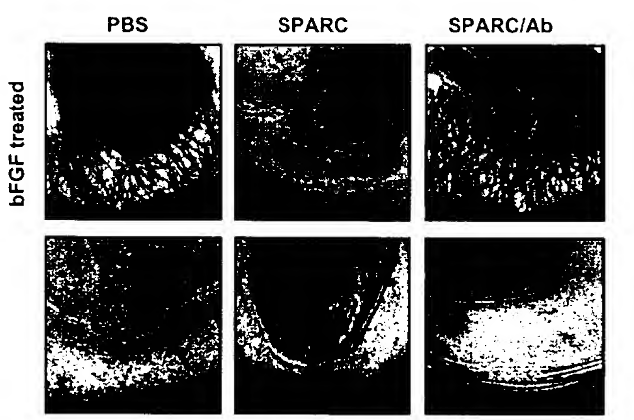


Fig. 4. SPARC inhibits angiogenesis *in vivo*. *Top panels*, SPARC inhibits bFGF-induced angiogenesis in the rat corneal assay. Addition of anti-SPARC antibody restored angiogenesis by bFGF. *Bottom panels*, in the absence of bFGF, control media, purified SPARC, or purified SPARC plus anti-SPARC neutralizing antibody did not induce angiogenesis in rat corneal assays.

tumors doubled every 5–6 days (Fig. 5, *A* and *B*). During the third week of treatment, a slight increase in tumor size was observed in the treatment group. After 3 weeks of treatment with SPARC, the average tumor volume was significantly smaller than that observed in control animals (152 ± 44 mm^3 *versus* 919 ± 317 mm^3; $P = 0.03$). Histological comparison revealed decreased vascularity in SPARC-treated tumors (MVD = 23/mm^2) compared with control tumors (MVD = 47/mm^2) as assessed by the number of structures that stained positively with an anti-CD31 antibody (Fig. 5, *C* and *D*).

DISCUSSION

Schwann cells secrete substances that promote NB cell survival and differentiation and inhibit angiogenesis (25, 27, 29, 30). It is thought that cross-talk between neuroblasts and Schwann cells is responsible for the more benign nature of Schwannian stroma-rich/stroma-dominant NB tumors (25, 29). Seeking factors produced by Schwann cells that could contribute to this clinically less aggressive tumor phenotype, we identified a factor present in SCM that was capable of inhibiting angiogenesis.

Table 1 *In vivo antiangiogenic activity of SPARC*

Sample	bFGF	Positive corneas/total implanted
PBS	No	0/6
PBS	Yes	9/9
SPARC	No	1/7
SPARC	Yes	0/8
SPARC antibody	No	0/5
SPARC antibody	Yes	7/7

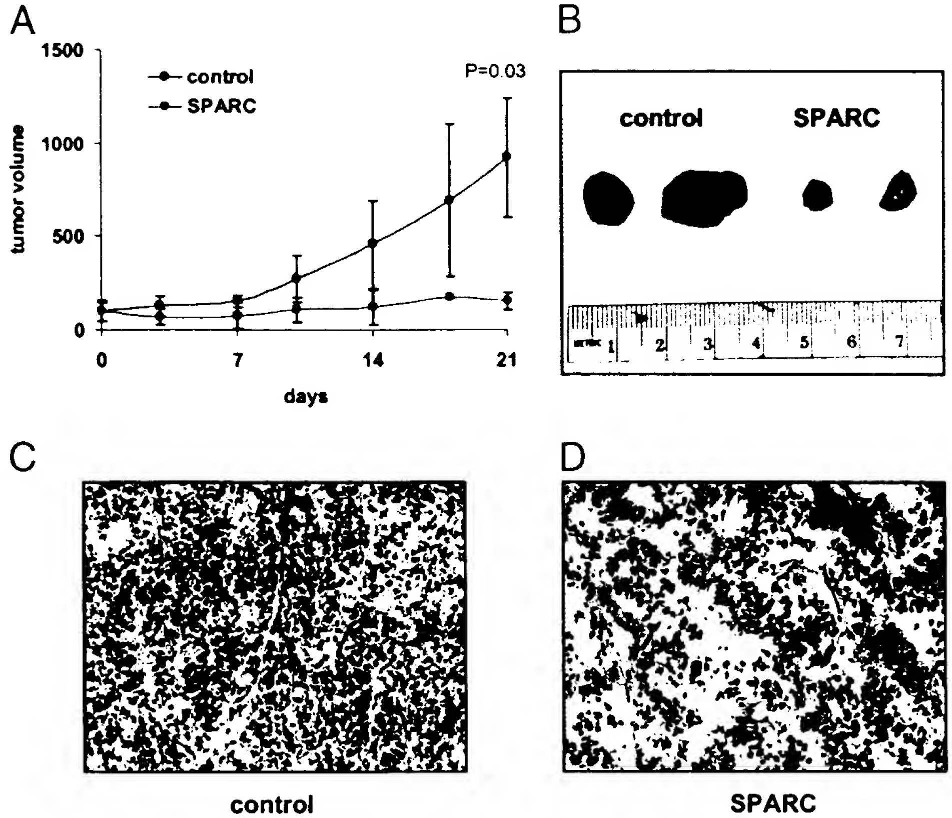


Fig. 5. SPARC inhibits NB tumor growth *in vivo*. *A* and *B*, short-term delivery of SPARC by osmotic pumps impairs the rate of NB xenograft tumor growth compared with control animals. *C* and *D*, immunohistochemistry staining of the SMS-KCNR NB xenograft tumors with anti-CD31 antibody demonstrates decreased numbers of blood vessels in NB xenografts from the SPARC-treated mice compared with control-treated animals (magnification, ×200).

SPARC, a known member of the matricellular protein family, appears to be a key contributor to the cumulative antiangiogenic activity produced by Schwann cells. We confirmed a known ability of SPARC to block chemotaxis of endothelial cells induced by angiogenic stimuli (38) and report its previously unknown function to induce endothelial cell apoptosis.

Previous studies have indicated that SPARC contributes to the regulation of tumor formation, although its role appears to be cell type specific. SPARC expression is down-regulated in rat and chick embryo fibroblasts transformed with c-Jun and v-Src (40, 62), and its reintroduction counteracts tumorigenesis by these cells (41). In addition, SPARC slows *in vitro* growth, induces apoptosis, and reduces tumorigenicity of ovarian cancer cells (44). In contrast, suppression of SPARC expression abrogates the tumorigenicity of melanoma cells (42).

SPARC is also involved in angiogenesis. SPARC blocks the G_1 to S transition of endothelial cells manifested by decreased proliferation rates (63, 64) and antagonizes the activity of the potent angiogenic activators bFGF and VEGF (38, 39). SPARC-null mice display substantially higher tumor invasion and angiogenesis compared with their wild-type littermates (65), indicating that SPARC may interfere with tumor angiogenesis. However, other studies point to proangiogenic activity of SPARC (66): it was found at high levels in breast cancer and colon cancer (67, 68), in metastatic melanoma (69), and in invasive meningiomas (70). Our studies suggest that disparate data regarding the role of SPARC in tumor growth and angiogenesis may be explained by its biphasic effect on the endothelial cells, where higher concentrations appear inactive or even stimulatory. Such biphasic effects are not uncommon for antiangiogenic factors and are shared by at least two more inhibitors, thrombospondin-1 (60, 61) and PEDF.[4,5]

Our study revealed an inverse correlation between SPARC expression levels and the degree of malignant progression in NB tumors. Whereas tumorigenic cell lines and N-type subclones showed low or no detectable levels of SPARC mRNA and secreted protein, nontumorigenic S-type NB subclones and NB cells induced to differentiate *in vitro* expressed SPARC at high levels. In maturing and mature NB tumors rich in Schwannian stroma, Schwann cells and differentiated neuroblasts/ganglion cells showed strong positive staining for SPARC, whereas little to no staining was detected in undifferentiated, Schwannian stroma-poor tumors. Furthermore, we demonstrated antiangiogenic activity of SPARC in a mouse NB xenograft model.

The mechanisms underlying these highly variable cell type-specific activities of SPARC remain unknown. However, similar to thrombospondin-1 (60, 61), concentration-dependent activity of SPARC may be explained if two distinct receptors are present on vascular endothelium, where a low-affinity receptor activated by higher ligand concentrations conveys a proangiogenic function of SPARC, and a high-affinity receptor is antiangiogenic. SPARC function may also be altered via posttranslational modifications. SPARC subspecies secreted by normal fibroblasts and by melanoma tumors differ in size and glycosylation pattern (69). It is tempting to speculate that turnover rates and receptor affinity of SPARC produced by malignant melanoma cells may be disparate from those of SPARC from normal stroma. Specific cleavage of SPARC by tumor cells may be glycosylation dependent and lead to an altered function. This hypothesis appears more feasible because the peptides from distinct structural domains of SPARC affect diverse cell phenotypes including growth rate, cell shape, matrix attachment, and angiogenic potential (66, 69, 71). Changes in any of these functions alone may lead to decreased

[4] D. W. Dawson, unpublished data.

[5] R. Schodlu *et al.* Augmentation of choroidal neovascularization in a laser induced mouse model by PEDF, in preparation.

angiogenesis and slow down tumor progression. However, it is likely that it is the ability of SPARC, shared with the majority of the angiogenesis inhibitors (reviewed in Refs. 72 and 73), to induce apoptosis in the endothelial cells of remodeling vasculature that allows it to interrupt angiogenesis and to delay tumor progression.

Our data point to SPARC as a key contributor to the antiangiogenic activity of factors secreted by the Schwann cells. However, other inhibitors of angiogenesis including PEDF and TIMP-2 also appear to be involved in the cross-talk between Schwann cells and the neuronal component of NB tumors (29, 30, 74). Our data clearly demonstrate that the angiogenic balance is complex: it is not a result of a single inducer-inhibitor combination but a composite value determined by all of the inducers and inhibitors present (reviewed in Ref. 75). Changes in angiogenic phenotype may occur gradually (76) or in a single step (77, 78). In Schwannian stroma-rich/stroma-dominant NB tumors, a spectrum of angiogenic inhibitors appears to be important for maintaining the net inhibitory phenotype, possibly because the nature of the angiogenic balance in NB tumors is quite complex. In addition to its effect on angiogenesis, PEDF also promotes survival and differentiation of the neuronal component of the tumors and thus creates a positive feedback loop (30), whereas TIMP-2 suppresses tumor cell invasion (79). A better understanding of the complex regulation of NB angiogenesis will hopefully lead to the development of new therapies in which inhibitors of angiogenesis may be used in concert to improve the outcome of children with clinically aggressive, Schwannian stroma-poor NB.

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TARGETING ANGIOGENESIS INHIBITS TUMOR INFILTRATION AND EXPRESSION OF THE PRO-INVASIVE PROTEIN SPARC

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The solid growth of high-grade glioma appears to be critically dependent on tumor angiogenesis. It remains unknown, however, whether the diffuse infiltration of glioma cells into healthy adjacent tissue is also dependent on the formation of new tumor vessels. Here, we analyze the relationship between tumor angiogenesis and tumor cell infiltration in an experimental glioma model. C6 cells were implanted into the dorsal skinfold chamber of nude mice, and tumor angiogenesis was monitored by intravital fluorescence videomicroscopy. Glioma infiltration was assessed by the extent of tumor cell invasion into the adjacent chamber tissue and by expression of SPARC, a cellular marker of glioma invasiveness. To test the hypothesis that glioma angiogenesis and glioma infiltration are codependent, we assessed tumor infiltration in both the presence and the absence of the angiogenesis inhibitor SU5416. SU5416 is a selective inhibitor of the VEGF/Flk-1 signal-transduction pathway, a critical pathway implicated in angiogenesis. Control tumors demonstrated both high angiogenic activity and tumor cell invasion accompanied by strong expression of SPARC in invading tumor cells at the tumor–host tissue border. SU5416-treated tumors demonstrated reduced vascular density and vascular surface in the tumor periphery accompanied by marked inhibition of glioma invasion and decreased SPARC expression. A direct effect of SU5416 on glioma cell motility and invasiveness was excluded by *in vitro* migration and invasion assays. These results suggest a crucial role for glioma-induced angiogenesis as a prerequisite for diffuse tumor invasion and a possible therapeutic role for anti-angiogenic compounds as inhibitors of both solid and diffuse infiltrative tumor growth. *Int. J. Cancer* 87:261–268, 2000.

High-grade gliomas represent the most frequent and malignant astroglial tumors. Due to their resistance to conventional oncological therapy, the mean survival time for patients with high-grade gliomas is approximately 9 to 12 months. Glioma pathology is characterized by both solid and diffuse infiltrative growth patterns. A strong line of experimental evidence supports the concept that solid glioma growth is critically dependent on angiogenesis, *i.e.*, the formation of new blood vessels that guarantee sufficient tissue oxygenation and nutritional supply (Millauer *et al.*, 1994). In addition, tumor vessel density has become an independent prognostic marker in human astroglial tumors (Leon *et al.*, 1996). In contrast, the diffuse, infiltrative tumor component is characterized by aggressive invasion of single tumor cells into the surrounding morphologically and functionally intact brain tissue. It is this diffuse cell invasion that finally determines the malignancy of high-grade glioma and is responsible for the inevitable tumor recurrence following radical surgical resection. It remains unknown whether this diffuse, infiltrative growth of gliomas is similarly angiogenesis-dependent.

Vascular endothelial growth factor (VEGF) is the principal angiogenic growth factor in experimental and human astroglial tumors (Plate *et al.*, 1993). VEGF exerts its biological effects by 2 high-affinity receptors, the class III protein tyrosine kinases VEGFR-1 [fms-like tyrosine kinase-1 (Flt-1)] and VEGFR-2 [fetal liver kinase-1 (Flk-1)/kinase insert domain–containing receptor], which are almost exclusively expressed on microvascular endothelial cells. Based on extensive work with experimental gliomas such as intracerebral and s.c. C6 glioma xenografts, VEGF/Flk-1 has been implicated as the dominant signal-transduction pathway mediating glioma angiogenesis *in vivo* (Plate *et al.*, 1993; Millauer *et al.*, 1994). As a consequence, targeting Flk-1 has become of considerable interest as an alternative anti-tumor therapy, and a novel selective inhibitor of the catalytic tyrosine kinase activity of Flk-1, SU5416, has been identified (Fong *et al.*, 1999). *In vitro* studies revealed a potent anti-proliferative effect of SU5416 on endothelial cells without directly affecting tumor cell proliferation (Fong *et al.*, 1999). Our intravital microscopic studies using the C6 glioma cell line and the dorsal skinfold chamber preparation in nude mice have shown that solid tumor growth suppression by SU5416 is the result of its direct inhibitory effect on tumor-induced angiogenesis and microvascular proliferation (Vajkoczy *et al.*, 1999).

SPARC (secreted protein acidic and rich in cysteine) is a developmentally regulated, glycosylated, anti-adhesive protein that is secreted into the extracellular matrix (ECM) (Lane and Sage, 1994). It is proposed to modulate cell migration and vascular morphogenesis either by directly interacting with ECM proteins or by initiating a receptor-mediated signaling event (Murphy-Ullrich *et al.*, 1995). SPARC interacts with several brain ECM collagens (Sage, 1997) and binds directly to vitronectin (Rosenblatt *et al.*, 1997), a multifunctional adhesive protein that is a component of brain vascular basement membranes. SPARC induces intracellular changes of cytoplasmic components associated with focal adhesions (Murphy-Ullrich *et al.*, 1995), including the phosphorylation of β-catenin and paxillin (Young *et al.*, 1998), the redistribution of vinculin from a punctate to a diffuse localization (Sage, 1997) and the redistribution of F-actin to the cell periphery (Sage, 1997). Thus, SPARC may directly affect cell motility. SPARC may also indirectly promote cell migration by modulating the expression of proteolytic enzymes that degrade the ECM, such as collagenase, stromelysin and MMP-9 (Tremble *et al.*, 1993). Our studies have demonstrated increased SPARC expression in infiltrating tumor cells at the brain–tumor interface in gliomas, indicating that

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SPARC may promote diffuse tumor cell infiltration into adjacent brain by affecting both tumor- and endothelial cell-ECM interactions and may therefore serve as a cellular marker of the invasive glioma phenotype (Rempel *et al.*, 1998).

Our objective was to test the hypothesis that diffuse, infiltrative glioma growth is dependent on glioma-induced angiogenesis. If these biological processes were codependent, specific inhibition of tumor angiogenesis should impact both tumor invasion and expression of SPARC by tumor cells. Therefore, we examined tumor angiogenesis, tumor cell infiltration and SPARC expression in the absence and presence of SU5416 in a C6 glioma xenograft. This tumor model was chosen because the tumor behavior closely approximates that of intracerebral human high-grade gliomas such as glioblastoma multiforme. Furthermore, it has been extensively characterized with respect to the functional expression of VEGF and Flk-1 *in vivo* (Plate *et al.*, 1993), tumor-induced angiogenic activity (Vajkoczy *et al.*, 1999) and diffuse, infiltrative growth behavior (Bernstein and Woodward, 1995). Our results indicate that angiogenesis not only plays a critical role in solid glioma growth but also is critically involved in diffuse, infiltrative glioma growth.

MATERIAL AND METHODS

Cells and cell culture

C6 rat glioma cells were frozen in Ham's F-10 culture medium with 10% DMSO. Thawed cells were grown in Ham's F-10 culture medium and plated in 12-well dishes for incubation in a 5% CO_2 humidified atmosphere at 37 C. Prior to tumor inoculation, C6 glioma cells were fluorescently labeled with the neuronal tracer Fast Blue (Sigma, St. Louis, MO), as previously described (Vajkoczy *et al.*, 1999).

Animals and dorsal skinfold chamber model

Athymic nude mice (n = 12, *nu/nu*, male, 30 to 35 g) were bred and maintained within a specific pathogen germ-free environment. As implantation site for the C6 glioma cells, we used the dorsal skinfold chamber preparation that contains 1 layer of striated muscle, s.c. tissue and epidermis (Vajkoczy *et al.*, 1999). This model is characterized by both solid and diffuse infiltrative glioma growth patterns and represents a versatile technique for the non-invasive analysis of glioma microcirculation and hemodynamics (Vajkoczy *et al.*, 1999).

Experimental protocol

The study was approved by the animal care and use committee of the local government authorities. Starting on the day of glioma cell implantation, 4 animals received a daily i.p. bolus of SU5416 [25 mg/kg body weight dissolved in 50 µl DMSO]. As controls, 4 animals received either DMSO (50 µl i.p.) or PBS (50 µl, i.p.) daily and were analyzed. The dose of SU5416 was selected by toxicity studies (Fong *et al.*, 1999) that identified 25 mg/kg daily as the maximal tolerated dose that does not exhibit significant toxicity in the mouse. Tumor-induced angiogenesis was assessed by intravital fluorescence videomicroscopy on days 6, 10, 14, 18 and 22 after cell implantation (see below). At the end of the observation period (day 22), animals were killed and tumors further processed for quantitative, histomorphological and immunohistochemical analyses (see below). To evaluate the effect of tumor size and, thus, total tumor cell number on the extent of glioma cell invasion, 4 additional control glioma tumors with a volume comparable to day 22 SU5416-treated tumors were assessed histologically on day 10 after cell implantation.

Intravital fluorescence videomicroscopy

The glioma mass and its new microvasculature were analyzed by intravital fluorescence videomicroscopy (epi-illumination) using a modified Axiotech Vario microscope, Zeiss, Oberkochen, Germany with UV (340 to 380 nm) and blue (450 to 490 nm) filter blocks (Vajkoczy *et al.*, 1999). Individual microvessels and the glioma microvasculature were visualized by contrast enhancement with FITC-conjugated dextran (Vajkoczy *et al.*, 1999). All microcirculatory measurements were made using a computer-assisted image analysis system (CAPIMAGE; Zeintl Software Engineering, Heidelberg, Germany). Tumor volume (mm^3) was calculated using the equation $V = 2/3 \times A \times h$, where A is the area covered by the fluorescently labeled glioma mass within the chamber (mm^2) and h is the thickness of the glioma bulging out at the chamber backside (mm). For microcirculatory analysis, the newly formed microvasculature of the glioma mass was divided into 3 radial zones (peri-tumoral, marginal and central). Each zone was assessed separately (2 to 3 observation fields/zone), resulting in a total of 6 to 9 measurements per animal. Quantitative analysis included vascular density (cm^{-1}), which was defined as the length of all newly formed microvessels per area of interest and observation time point, and the vascular surface, which was analyzed in relation to the total area of interest (%).

Histology and immuno-histochemistry

For histomorphological analysis, tumor-containing dorsal skinfold chamber preparations were excised, fixed over 48 hr using 4% paraformaldehyde and embedded in paraffin. The technical setup for histomorphological analyses included an Olympus (Tokyo, Japan) microscope with ×10, ×20 and ×40 objectives; a low-light level, charge-coupled device video camera (Cohu FK 6990; Pieper, Schwerte, Germany); and an S-VHS video system (Panasonic, Munich, Germany). Images were analyzed off-line using the computer-assisted image analysis system described previously. For conventional histology 5 µm serial sections were stained with hematoxylin–eosin. SPARC immuno-histochemical analysis was performed as previously reported (Rempel *et al.*, 1998) with minor changes. Briefly, formalin-fixed, paraffin-embedded 5 µm tissue sections were subjected to routine deparaffinization and rehydration. Subsequent steps were performed at room temperature unless otherwise specified. Sections were incubated for 10 min in 3% hydrogen peroxide, rinsed in PBS solution and incubated with 10% horse serum in PBS containing 0.3% Triton X-100 (TPBS) for 60 min. Sections were incubated overnight at 4°C with a 1:900 dilution (5.7 µg/ml) of primary anti-SPARC antibody (Hematologic Technologies, Essex Junction, VT) in TPBS. After 3 washes in TPBS buffer, sections were incubated for 30 min with biotinylated secondary antibody (1:400 dilution in TPBS), washed and incubated for 45 min with avidin–biotin complex according to the manufacturer's instructions (Vectastain ABC kit; Vector, Burlingame, CA). Finally, sections were washed, reacted with diaminobenzidine (DAB) in 0.1 M Tris buffer (pH 7.6) with 0.03% hydrogen peroxide, rinsed in tap water, counterstained and mounted. Controls were performed by omitting the primary anti-SPARC antibody or by substituting the primary antibody with mouse IgG isotype. Staining was noted as either positive or negative. Tumor cells were identified by histomorphology (*i.e.*, rounded cells with large and hyperchromatic irregular nuclei) and detection of the glial marker S-100 protein. For S-100 protein staining, 5 µm tissue sections were subjected to citric acid antigen retrieval as previously described for SPARC immuno-histochemistry (Rempel *et al.*, 1998). S-100 protein was detected using a 1:300 dilution of rabbit anti-cow S-100 (Z0311; Dako, Carpinteria, CA) that cross-reacts strongly with human S-100A and -B and is useful in distinguishing brain glial cell tumors.

Tumor cell invasion

Invasion of glioma cells (*i.e.*, S-100 protein–expressing cells) into the adjacent chamber tissue was assessed by semi-quantitative and linear means. Thus, the density of infiltrating tumor cells was rated as none (–), minimal (+), moderate (++) or high (+++). For linear analysis (×10 objective), the length of the infiltrated and destroyed (*i.e.*, complete infiltration of tissue and loss of characteristic histomorphology) muscle and s.c. tissue (mm) was measured. Results were expressed as percentage of the total cross-sectional length of the glioma mass.

In vitro tumor cell migration and invasiveness

To assess the direct, angiogenesis-independent effects of SU5416 on glioma cell migration and invasion, the *in vitro* spheroid migration and spheroid confrontation assays were employed. Fetal rat brain spheroids and glioma spheroids represent standardized, primary, avascular brain and tumor masses that resemble brain and glioma tissues *in situ*, thus providing a suitable model to investigate glioma cell migration and vascular cell-independent invasion *in vitro* (Tonn *et al.*, 1995). C6 spheroids were produced by seeding 5×10^6 cells into a 75 cm^2 flask previously coated with 1.0% Noble agar (Difco, Detroit, MI) covered by a liquid medium overlay. Spheroids were screened for any sign of necrosis by inverted light microscopy. Necrotic cores were observed in spheroids with diameters >400 μm. Accordingly, after 7 to 10 days, 300 μm spheroids were chosen for migration and invasion studies. Brain cell aggregates were generated according to a standardized procedure described previously (Tonn *et al.*, 1995). C6 spheroids were placed in the middle of poly-D-lysine–coated 24-well plates. Spheroid growth was assessed in medium that contained PBS, 0.1% DMSO, or SU5416 at 0.1 μM and 1 μM. For the migration assay, the medium contained SU5416 dissolved in 0.1% DMSO at the following concentrations: 0.1, 1, 10 and 100 μM. Medium containing PBS or 0.1% DMSO alone served as control. The area covered by cells spreading out from the spheroid was measured using an image analysis system (Intas, Göttingen, Germany) after 24 and 48 hr. For the confrontation assay, single mature brain aggregates (diameter 250 to 350 μm) were placed into agar-coated well plates. Single gliomas of similar size were also transferred into the wells and brought in contact with the brain aggregates. SU5416-containing medium was added at the same concentrations used for the migration assays, and medium containing PBS or 0.1% DMSO alone served as control. Over a 48 hr period of confrontation, microphotographs were taken every 24 hr and evaluated semi-quantitatively using the Intas image analysis system. Glioma cell invasion was assessed for the amount of rat brain spheroid remaining intact. All proliferation, migration and confrontation assays were performed in quadruplicate.

Statistical analysis

Quantitative data are given as mean ± SEM. Mean values of microcirculatory and histomorphological data were calculated from the average values in each animal. For analysis of differences between groups, 1-way ANOVA followed by unpaired Student's *t*-test was performed. Results with $p < 0.05$ were considered significant. Since no significant difference was measured in any of the evaluated parameters between control animals receiving DMSO or PBS, these were grouped together for graphic representation and statistical evaluation.

RESULTS

Tumor angiogenesis

Implantation of C6 glioma cells into the dorsal skinfold chamber induced a strong angiogenic response. The tumor microvasculature demonstrated chaotic micro-angioarchitecture, heterogeneous blood perfusion and high microvascular permeability for the fluorescent marker FITC-dextran (Fig. 1*a*). Quantitative analysis of the microcirculatory parameters revealed high vascular density and vascular surface in the tumor periphery with a progressive reduction toward the tumor center (Fig. 1*b,c*). Treatment with SU5416 resulted in marked suppression of glioma-induced angiogenesis with significant reduction of vascular density when compared to controls (Fig. 1*b*). Zonal microvascular analysis revealed that this anti-angiogenic effect was most pronounced in peri-tumoral areas (*i.e.*, the more angiogenic and better vascularized peripheral areas). Since blocking angiogenesis was not accompanied by significant changes of the microvascular diameters, a similar pattern was observed with respect to the vascular surface (Fig. 1*c*).

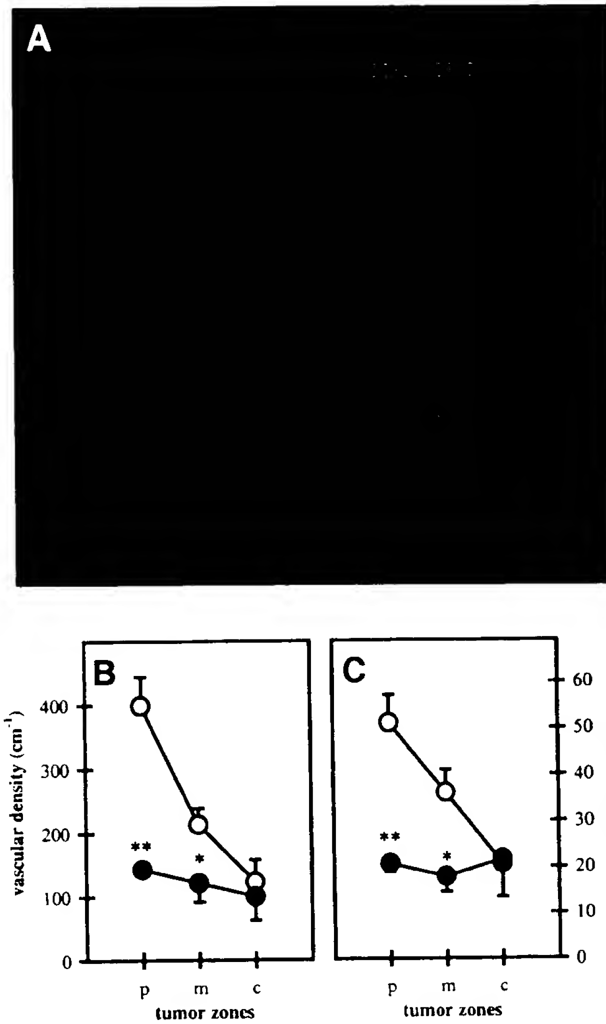

FIGURE 1 – Microvasculature of control and SU5416-treated C6 gliomas. *(a)* Microvasculature of control tumor (50 μl DMSO/day i.p.) on day 10 after cell implantation, assessed by intravital fluorescence videomicroscopy and contrast enhancement with 2% FITC-dextran$_{150}$ i.v. Scale bar = 50 μm. *(b,c)* Influence of Flk-1 tyrosine kinase inhibition on C6 glioma-induced angiogenesis after cell implantation into the dorsal skinfold chamber of nude mice (day 18). Vascular density *(b)* and vascular surface *(c)* were quantitatively assessed within peri-tumoral (p), marginal (m) and central (c) areas of the tumor. Animals were treated with PBS/DMSO (50 μl/day i.p., n = 4, open symbols) or SU5416 (25 mg/kg body weight/day i.p., n = 4, closed symbols). Vascular density and vascular surface were analyzed offline using a computer-assisted image analysis system. Mean ± SEM values are represented. Statistical analysis was performed using ANOVA followed by unpaired Student's *t*-test. $*p < 0.05$, $**p < 0.01$ *vs.* PBS/DMSO.

Solid tumor growth

Suppression of glioma-induced angiogenesis was accompanied by a reduction of solid glioma growth. On days 10 and 22 after glioma cell implantation, the volume of control tumors measured 36.2 ± 2.9 mm^3 and 433.3 ± 5.5 mm^3, respectively. In contrast, the volume of SU5416-treated tumors measured 1.2 ± 0.7 mm^3 and 36.1 ± 8.1 mm^3 on days 10 and 22 after glioma cell implantation ($p < 0.01$), respectively.

Diffuse infiltrative tumor growth

Apart from its inhibitory effect on solid glioma growth, suppression of tumor angiogenesis resulted in marked reduction of

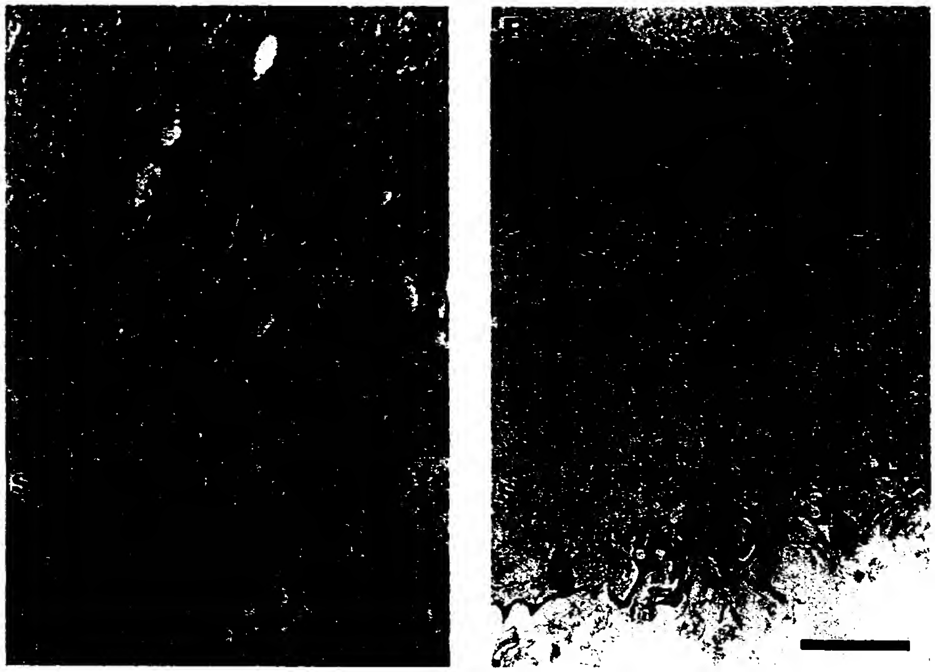

FIGURE 2 – Histological examination of C6 gliomas on day 22 after glioma cell implantation into dorsal skinfold chamber of nude mice treated with either *(a)* DMSO (50 μl daily i.p.) or *(b)* SU5416 (25 mg/kg body weight daily i.p.). *(a)* Glioma cell invasion into underlying striated muscle and s.c. tissue with subsequent formation of large tumor masses. Note loss of histomorphological integrity due to tissue destruction. *(b)* Near-complete suppression of glioma cell invasion. Tumor mass is well delineated from muscle and s.c. tissue by intact basement membrane. t, tumor mass; m, striated muscle; s, s.c. tissue. Hematoxylin and eosin staining of 5 μm sections. Scale bar = 500 μm.

diffuse glioma cell invasion into the adjacent tissue. To evaluate the effect of SU5416 on diffuse, infiltrative glioma growth, control and treated tumors were analyzed for tumor cell invasion into the underlying muscle and s.c. tissue. Histomorphological analysis of control specimens demonstrated strong (+++) tumor cell infiltration on day 22 (Fig. 2*a*). In contrast, specimens of SU5416-treated animals showed a near-complete reduction of glioma cell invasion into the adjacent host tissue. By day 22, only individual tumor cells could be identified within the intact muscle and s.c. tissue (+) (Fig. 2*b*). Quantitative analysis of control specimens revealed that by day 22 tumor cells had invaded most of the adjacent muscle and s.c. tissue (Fig. 3*a*). More than 60% of the length of muscle and s.c. tissue revealed maximum single glioma cell invasion resulting in tissue destruction with loss of histological integrity. Treatment with SU5416 reduced the length of muscle and s.c. tissue infiltration by 33% and 50%, respectively (Fig. 3*b*). In these specimens, formation of infiltrating tumor masses with destruction of the adjacent tissue was not observed.

To rule out that this suppression of infiltrative tumor growth was simply due to a reduction in tumor volume and, thus, tumor cell burden, we additionally analyzed similarly sized control day 10 tumors (volume 36.2 ± 2.9 mm^3) and SU5416-treated day 22 tumors (volume 36.1 ± 8.1 mm^3). Even in these small tumors, glioma cell infiltration into the adjacent host tissue was more advanced than in SU5416-treated tumors (++) (Fig. 3*c*).

Relationship between tumor angiogenesis and tumor infiltration

The relationship between tumor angiogenesis and tumor cell invasion into the adjacent tissue was further analyzed. The mean vascular density within the peri-tumoral area was compared to the density of tumor cells within the adjacent chamber tissue. As illustrated in Figure 4, glioma cell invasion was strongly related to glioma-induced peri-tumoral angiogenic activity. This relationship was independent of tumor size and time of analysis after cell implantation.

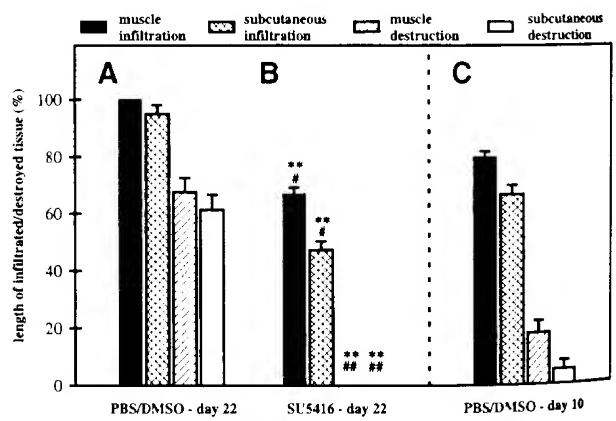

FIGURE 3 – Influence of Flk-1 tyrosine kinase inhibition on the extent of glioma cell invasion into adjacent tissue on day 22 after cell implantation. Linear analysis of length of infiltrated and destroyed muscle and s.c. tissue as percentage of total cross-sectional length of tumor mass. Animals were treated with *(a)* PBS/DMSO (50 μl daily i.p., n = 4) or *(b)* SU5416 (25 mg/kg body weight daily i.p., n = 4) for 22 days. *(c)* Additional PBS/DMSO-treated animals (50 μl daily i.p., n = 4; right bars) were assessed after 10 days, to analyze the impact of tumor volume/tumor cell number on glioma cell invasion. Mean ± SEM values are represented. Statistical analysis was performed using ANOVA followed by unpaired Student's *t*-test. $^{**}p < 0.01$ *vs.* PBS/DMSO, day 22; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ *vs.* PBS/DMSO, day 10.

Effect of SU5416 on tumor cell migration and invasion in vitro

To determine whether the *in vivo* suppression of glioma cell infiltration into the adjacent host tissue was due to a direct inhibitory effect of SU5416 on glioma cell motility and invasiveness or to the anti-angiogenic activity of the compound, we performed *in*

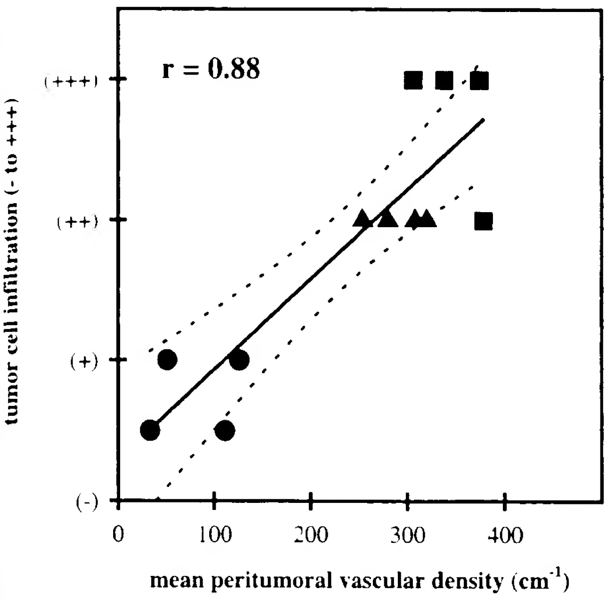

FIGURE 4 – Relationship between peri-tumoral vascular density and density of glioma cells in adjacent chamber tissue. Peri-tumoral vascular density is given as mean of values obtained prior to analysis of tumor infiltration. Density of S-100 protein–expressing cells in adjacent muscle tissue was assessed in controls on day 10 (triangles) as well as in controls (squares) and treated animals (circles) on day 22 after cell implantation. Animals were treated with PBS/DMSO (50 µl daily i.p., n = 4 each, triangles and squares) or SU5416 (25 mg/kg body weight daily i.p., n = 4, circles). Vascular density was analyzed off-line using a computer-assisted image analysis system. Tumor cell density was assessed semi-quantitatively: –, no infiltration; +, minimal infiltration; ++, moderate infiltration; +++, strong infiltration. Regression analysis with 95% confidence interval (dotted line).

vitro proliferation, migration and confrontation assays with C6 glioma spheroids exposed to various concentrations of SU5416. SU5416 did not affect the viability or growth of C6 glioma spheroids (Fig. 5*a*). A dose-response curve could be generated for the effect of SU5416 on the directional migration of C6 glioma cells. No measurable effect was observed at concentrations below 10 µM (Fig. 5*b*). At 10 and 100 µM, the area of cell migration was reduced by 12% and 61%, respectively, when compared with controls (Fig. 5*b*). In parallel, 48 hr treatment with SU5416 demonstrated no effect on invasion of C6 glioma spheroids into avascular brain tissue aggregates at concentrations of 0.1, 1 and 10 µM (Fig. 5*c–f*). Only a concentration of 100 µM resulted in a minimally reduced invasion zone (data not shown).

Based on these *in vivo* and *in vitro* studies, tumor angiogenesis and tumor infiltration were considered to be codependent in this experimental glioma model. These findings prompted us to further investigate whether tumor vascularization impacted not only the invasive behavior of individual tumor cells but also their expression of SPARC, a unique cellular marker for the invasive glioma phenotype.

SPARC expression during diffuse tumor infiltration

To first determine the expression pattern of SPARC relative to central, marginal and peri-tumoral tumor areas during diffuse infiltration, we performed immuno-histochemical analyses on control specimens by day 22 after implantation (Fig. 6). Within the central tumor area, SPARC expression was limited to scattered islands of cells (Fig. 6*a,b*, arrowhead in *a*). In contrast, SPARC was strongly expressed in tumor cells immediately adjacent to the muscle layer (Fig. 6*a,c*, large arrowhead in *a*), in tumor cells infiltrating through the muscle layer (Fig. 6*c*) and in nests of cells that had invaded into the s.c. region (Fig. 6*c,d*). Within the s.c. region, SPARC immuno-histochemical analysis revealed 2 populations of cells. One population included less cellularly dense nests of SPARC-expressing cells (Fig. 6*d,e*) immediately adjacent to populations of cellularly dense tumor cells that did not express SPARC (Fig. 6*d,e*). These SPARC$^-$ cells were tumor cells, as demonstrated by colocalization of S-100 to the same region on an adjacent section (Fig. 6*f*). (SPARC- and S-100 protein–non-expressing cells were classified as fibroblasts due to their spindle-shaped, hyperchromatic nuclei, having only small amounts of cytoplasm.)

SPARC expression following blockade of tumor angiogenesis

To determine the effect of blocking tumor angiogenesis on SPARC expression, we performed further immuno-histochemical analyses on SU5416-treated specimens by day 22 after implantation (Fig. 7). Within the reduced central tumor area (compare Figs. 7*a* and 6*a*), SPARC expression was limited to scattered individual cells post-SU5416 treatment (Fig. 7*a,b*, arrow). This was in contrast to the intra-tumoral nests of cells observed in the absence of the anti-angiogenic compound (compare Figs. 6*b* and 7*b*). In addition to these central tumor areas, marked differences in SPARC expression between treated and control animals were observed. At the glioma-adjacent tissue border, in regions where SU5416 treatment resulted in a complete blockade of tumor cell infiltration into the underlying muscle tissue, SPARC expression in tumor cells was negative (Fig. 7*c,d*). However, in the few regions where SU5416 failed to block tumor cell infiltration completely, focal SPARC expression could still be observed (Fig. 7*e,f*).

DISCUSSION

In accordance with our previous results, we have demonstrated that targeting of the VEGF/Flk-1 signal-transduction pathway, using the small-molecule Flk-1 tyrosine kinase inhibitor SU5416, suppresses glioma-induced angiogenesis and, consequently, solid glioma growth *in vivo*. The principal novel finding of our study is that this anti-angiogenic strategy additionally results in marked suppression of glioma cell invasion into adjacent tissue with concomitant decreased expression of the pro-invasive molecule SPARC. Thus, the suppression of angiogenesis might represent an effective means to control diffuse, infiltrative glioma growth and local tumor expansion.

The dorsal skinfold chamber used on an immunodeficient mouse strain is a well-established model to study implanted gliomas. This model permits non-invasive, repetitive and quantitative evaluation of tumor growth, tumor-induced angiogenesis and tumor microcirculation by intravital fluorescence videomicroscopy (Vajkoczy *et al.*, 1999). A further strength of this model is its high spatial resolution in the range of <1 µm, allowing for precise zonal analysis of the newly formed glioma microvasculature (Vajkoczy *et al.*, 1999).

Diffuse invasion of tumor cells into the adjacent brain tissue and subsequent tissue destruction is a hallmark of high-grade glioma biology and represents the major obstacle to successful surgical therapy. Accordingly, using an *in vivo* implantation model in the rat cerebral cortex, Chicoine and Silbergeld (1995) identified fluorescently labeled C6 cells in morphologically intact brain tissue >16 mm from the implantation site within 2 weeks after stereotactic cell implantation. This process of local invasion is defined as the active translocation of tumor cells across tissue boundaries and through host cellular and ECM barriers. In general, glioma cell invasion represents an active, multistep process involving proteolytic degradation of the ECM, receptor-mediated adhesion of tumor cells to matrix proteins and migration of single cells with the re-arrangement of membrane and cytoskeletal elements. However, despite a considerable experimental effort, the exact biomolecular mechanisms that trigger or facilitate glioma cell evasion from the tumor mass and subsequent invasion into the adjacent tissue remain elusive.

Previously, it was suggested that tumor cell invasion and angiogenesis might share common pathways requiring similar compo-

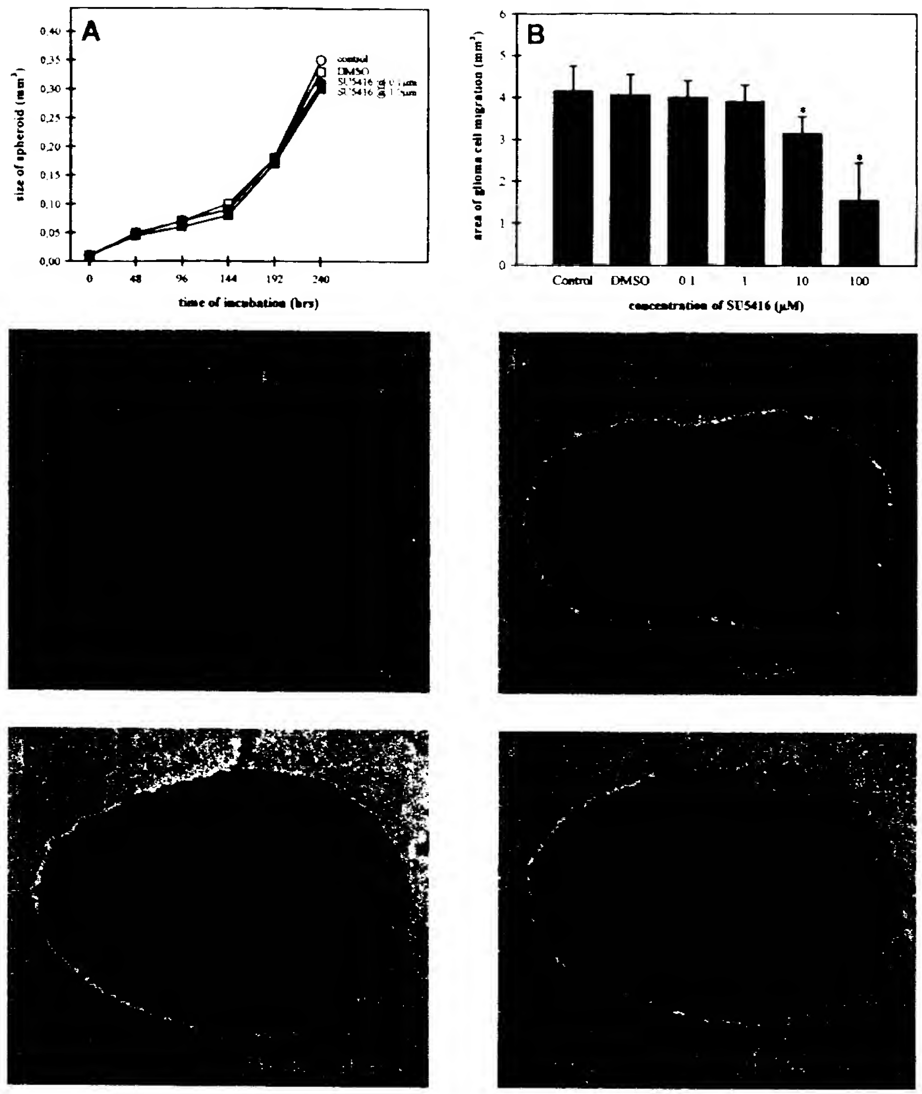

FIGURE 5 – Effect of SU5416 on *in vitro* C6 glioma spheroid proliferation, migration and invasion assays. *(a)* Proliferation of C6 glioma spheroids within 240 hr. Medium contained either PBS (control), 0.1% DMSO (DMSO) or SU5416 at 0.1 or 1 μM. Spheroid size was analyzed morphometrically by means of an image analysis system. Mean values are represented. *(b)* Migration of C6 glioma cells *in vitro* within 48 hr. Medium containing either PBS (control), 0.1% DMSO (DMSO) or SU5416 at various concentrations (0.1–100 μM). Area of migration was analyzed morphometrically by means of an image analysis system. Mean ± SD values are represented. Statistical analysis was performed using ANOVA followed by unpaired Student's *t*-test. $*p < 0.05$ *vs.* control. *(c–f)* Photomicrographs of C6 glioma spheroid (left) and fetal brain aggregate (right) in medium containing 0.1% DMSO (left column) and 10 μM SU5416 (in 0.1% DMSO) (right column), revealing unaffected glioma cell invasion after 24 and 48 hr of co-culture in the presence of SU5416. Toluidine blue staining.

nents of adhesion, proteolysis and cellular migration (Brooks *et al.*, 1994). Increased SPARC expression has been associated with both of these biological processes (Sage, 1997). Our results causally link glioma cell invasion and increased SPARC expression to glioma-induced angiogenesis. We have demonstrated that functional inactivation of Flk-1 by a selective small-molecule inhibitor of receptor tyrosine kinase activity suppresses not only glioma-induced angiogenesis but also glioma cell invasion into the adjacent tissue and SPARC expression. In addition, we have colocalized the major area of glioma-induced angiogenic activity, the major area of the anti-angiogenic activity of the Flk-1 tyrosine kinase inhibitor and the area where detached infiltrating tumor cells invade the adjacent host tissue.

This novel anti-invasive property of SU5416 cannot be explained by a direct effect of the angiogenesis inhibitor on glioma cell motility and invasiveness. Firstly, the expression and biological activity of Flk-1 are limited to endothelial cells (Millauer *et al.*, 1993). Secondly, SU5416 did not exert any direct inhibitory effect on glioma cell motility or invasiveness *in vitro* (*i.e.*, independent of tumor angiogenesis and vascularization) at concentrations that selectively suppress endothelial cell proliferation (IC_{50} = 0.4 μM) and that are pharmacologically relevant during daily systemic administration at a concentration of 25 mg/kg body weight. This is supported by HPLC measurements of SU5416 levels in plasma samples and C6 s.c. tumor extracts following i.p. administration of the compound, which revealed peak levels of 8.5 and 0.6 μM, respectively, and failed to demonstrate drug accumulation in the plasma or tumor tissue. The inhibitory effect of SU5416 on glioma cell motility at higher concentrations (>10 μM) is most likely attributable to weak cross-reactivity with the platelet-derived growth factor receptor (Fong *et al.*, 1999).

Based on these results, we propose that inhibition of glioma cell evasion from the tumor mass and/or glioma cell invasion into the adjacent tissue by SU5416 is the consequence of its anti-angio-

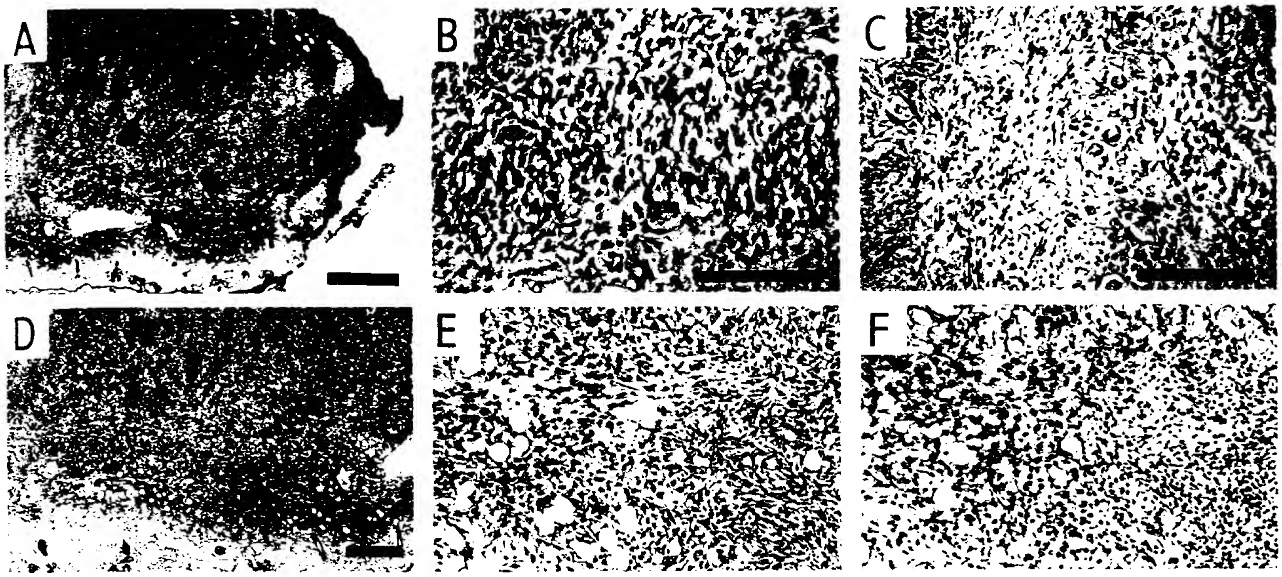

FIGURE 6 – SPARC expression during diffuse tumor infiltration (day 22). Animals were treated with DMSO (50 μl daily i.p.). *(a)* By day 22, SPARC expression is limited to islands of cells within the central part of the tumor (arrow) and expressed in cells in the marginal and peri-tumoral regions of the specimen (large arrowhead). Scale bar = 1 mm. *(b)* Magnification of the island of tumor cells in *(a, arrow)*. Scale bar = 200 μm. *(c)* Magnification of the central (C), marginal (M) and peri-tumoral (P) regions illustrated in *(a, large arrowhead)*. SPARC expression is observed in tumor cells immediately adjacent to the muscle layer, in tumor cells invading through the muscle layer and in cells in the peri-tumoral s.c. region. Scale bar = 100 μm. *(d)* Magnification of the region illustrated in *(a, small arrowhead)*. Scale bar = 200 μm. *(e)* Magnification of the region illustrated by the red asterisk in *(d)*. Two populations of cells are observed in the s.c. tissue, SPARC-expressing and SPARC–non-expressing cells. Magnification as in *(c)*. *(f)* Immuno-histochemical localization of S-100 protein. Cells negative for both SPARC *(e)* and S-100 protein *(f)* are identified as fibroblasts based on their histomorphological appearance. Magnification as in *(c)*.

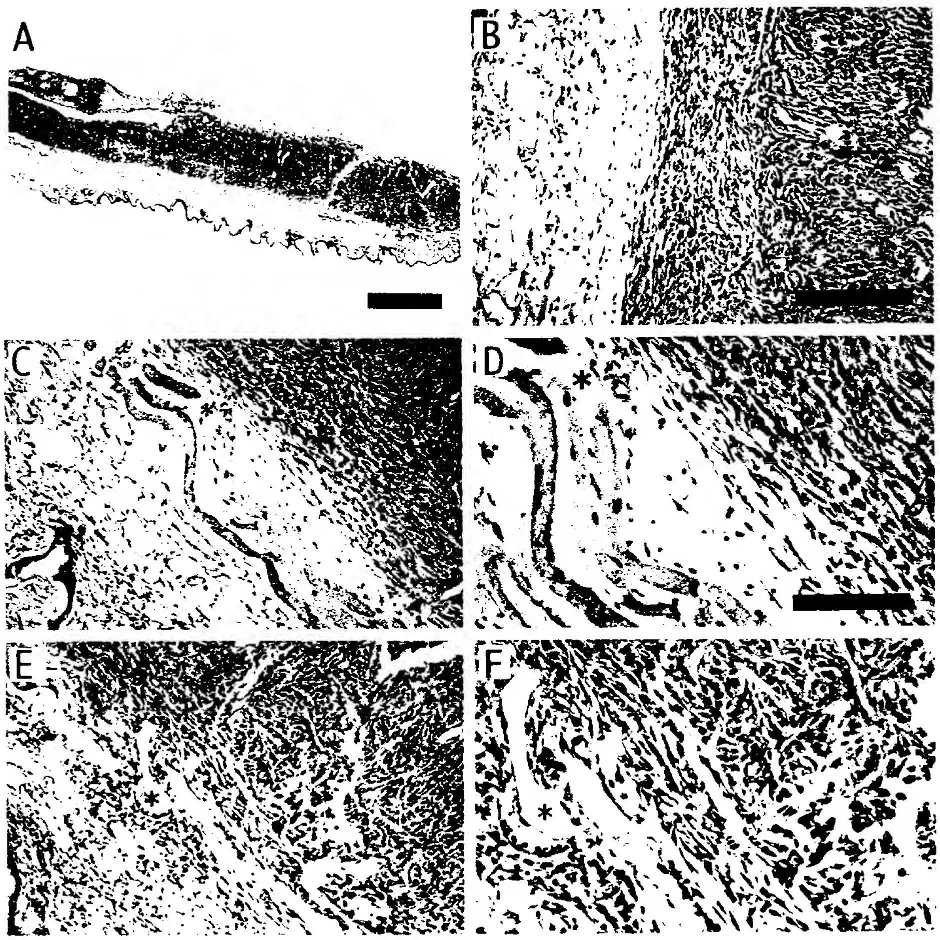

FIGURE 7 – SPARC expression during SU5416-induced inhibition of angiogenesis (day 22). *(a)* By day 22, growth of the tumor has been greatly inhibited (compare with Fig. 6*a*). Scale bar = 1 mm. *(b)* Magnification of region in *(a, red asterisk)*. SPARC expression within the central tumor area is limited to individual cells (arrow). Scale bar = 100 μm. *(c,d)* Magnification of the region in *(a, green asterisk)*. Scale bar = 200 μm. In regions where the inhibitor completely blocked invasion, SPARC expression was negative. *(e,f)* Magnification of the region in *(a, blue asterisk)*. In regions where the inhibitor did not completely block invasion, SPARC was observed in the infiltrating tumor cells.

genic activity. This is in line with other reports of suppression of malignant keratinocyte invasion by inactivation of Flk-1 using a blocking antibody (Skobe *et al.*, 1997).

One putative mechanism by which glioma-induced angiogenesis may facilitate glioma cell invasion is that new peripheral glioma microvessels which originate from the adjacent tissue and grow toward the tumor mass (Vajkoczy *et al.*, 1999) provide a trail for migrating and infiltrating glioma cells. This is in accordance with experimental results demonstrating that glioma cells are characterized by a high affinity for the peri-vascular basal membrane and, thus, seem to invade the adjacent tissue along peri-tumoral blood vessels (Giese and Westphal, 1996). A reduction of the vascular surface at the glioma-adjacent tissue border, as demonstrated in the present study (Fig. 2*b*), would thus result in a decreased space for the peri-vascular route of glioma cell infiltration.

Tumor infiltration is also facilitated by proteinases that are secreted by proliferating endothelial cells; *e.g.*, plasminogen activators (PAs) may facilitate degradation of the ECM and thereby allow tumor cells to invade adjacent tissue. This is relevant since urokinase-type PA (uPA) and its receptor (uPAR) have been identified in high-grade gliomas (Yamada *et al.*, 1994). Furthermore, proteinases can be up-regulated by angiogenic growth factors (*e.g.*, VEGF) *in vitro* (Pepper *et al.*, 1994). Finally, proliferating endothelial cells may influence the biological behavior of tumor cells through paracrine interactions by the secretion of growth and motility factors.

SPARC may contribute to several of these proposed mechanisms. Its interaction with the ECM molecule vitronectin (Rosenblatt *et al.*, 1997), found in high abundance along vessel basement membranes, may directly contribute to tumor invasiveness by facilitating migration along these structures. In addition, SPARC binds to the inhibitor of uPA (Rosenblatt *et al.*, 1997) and, thus, could indirectly promote tumor infiltration by permitting uPA degradation of the ECM. Furthermore, SPARC indirectly increases the expression of several MMPs (Tremble *et al.*, 1993), and this consequently could also facilitate cell migration. These data suggest that SPARC, either directly or indirectly, functionally contributes to cell migration/invasion and support the interpretation that the SPARC signal observed in C6 glioma cells identified invasive tumor cells. SPARC is also intimately involved in the regulation of angiogenesis through direct binding to VEGF (Kupprion *et al.*, 1998). Since SU5416 administration does not directly affect the ability of tumor cells to migrate *in vitro*, we propose that the inhibition of angiogenesis *in vivo* induces a putative anti-infiltration signal that directly or indirectly inhibits SPARC expression. Thus, the present data further demonstrate that tumor invasion and SPARC expression are closely associated and influenced by angiogenesis.

In conclusion, we propose that, at least in high-grade glioma, tumor-induced angiogenesis represents a crucial prerequisite not only for solid tumor growth but also for local cell invasion. If similar results are obtained for invasive tumors of other tissue origin, the diffuse, infiltrative tumor growth should be added to the list of angiogenesis-dependent tumor growth patterns that, to date, contains only solid tumor growth and metastatic spread of tumor cells *via* the circulatory system. Moreover, our data suggest a complex therapeutic role for the small-molecule Flk-1 antagonist SU5416 as an inhibitor of angiogenesis and both solid and diffuse infiltrative glioma growth patterns.

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Extracellular matrix components in intestinal development

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Abstract. Intestinal morphogenesis and differentiation are dependent on heterotypic cell interactions between embryonic epithelial cells (endoderm) and stromal cells (mesenchyme). Extracellular matrix molecules represent attractive candidates for regulators of these interactions. The structural and functional diversity of the extracellular matrix as intestinal development proceeds is demonstrated by 1) spatio-temporal specific expression of the classically described constituents, 2) the finding of laminin and collagen IV variants, 3) changes in the ratio of individual constituent chains, and 4) a stage-specific regulation of basement membrane molecule production, in particular by glucocorticoids. The orientation/assembly of these extracellular matrix molecules could direct precise cellular functions through interactions via integrin molecules.

The involvement of extracellular matrix, and in particular basement membrane molecules in heterotypic cell interactions leading to epithelial cell differentiation, has been highlighted by the use of experimental models such as cocultures, hybrid intestines and antisense approaches. These models allowed us to conclude that a correct elaboration and assembly of the basement membrane, following close contacts between epithelial and fibroblastic cells, is necessary for the expression of differentiation markers such as digestive enzymes.

Key words. Extracellular matrix; intestine; development; differentiation; interactions; integrins; hormones.

Introduction

Like many vertebrate organs, the digestive tract develops from heterogeneous embryonic anlagen. The epithelium derives from the endodermal germ layer, whereas the connective tissue and the musculature derive from the splanchnopleural mesenchyme.

The multistep process of intestinal morphogenesis and cell differentiation occurs in a precise species-specific temporal and spatial pattern. The sequences of structural and ultrastructural changes that take place in the epithelium during ontogenesis are closely similar in all species; only the timing of these processes is variable. For example, in rodents much of small intestinal development occurs late in the gestational period. This pattern differs from that of the human small intestine, which is well developed early in gestation. Whatever the species, morphogenetic and differentiation processes involved in small intestinal development include (see fig. 1) 1) formation of the primitive villi by folding of the embryonic endoderm, 2) progressive differentiation of endodermal cells into absorptive cells (also called enterocytes), goblet or mucus cells, and endocrine cells, 3) formation of crypts, at the base of villi, where dividing cells are then segregated at the perinatal period; a new specialized cell type appears, the Paneth cell which remains restricted to the bottom of the crypt, and 4) continuous maturation in the mature organ of the four main cell lineages from crypt to villus tip. During development as well as in the course of the crypt/villus maturation, differentiation of epithelial cells consists of morphological changes paralleled by the synthesis of specific proteins. The structural polarization of absorptive cells allows individualization of a specialized membrane domain, the apical brush border. This domain is composed of microvilli which are sustained by a highly organized cytoskeleton and endowed with functional proteins such as digestive enzymes (lactase, sucrase and peptidases) and transporters, implied in the terminal steps of carbohydrate/protein digestion and in absorption. Unlike the small intestine, the large intestine, or colon, is characterized by deep glands and a flat surface epithelium; its major function is to regulate fluid and electrolyte transport. A complete description of precise intestinal organogenesis is beyond the scope of this review and is well documented in detail in excellent reviews[62,70,83].

Ontogenic regulation by epithelium/mesenchymal interactions has been suggested for a number of organ systems. In the intestine, using several experimental models, it has been demonstrated that the mesenchyme plays a permissive role in the morphogenesis and cytodifferentiation of the digestive tract endoderm. This conclusion has been drawn by using, in particular, interspecies recombinants composed of chick and rat intestinal anlagen, in combination with species-specific morphological and biochemical analyses[65]. In addition, the potential involvement of reciprocal instructive interactions has been emphasized by the fact that, for example, intestinal mesenchyme was able to induce an intestinal cytodifferentiation of chick gizzard endoderm[48]. The presence of a sheath of fibroblasts underlying the crypt epithelium and migrating at least partly in

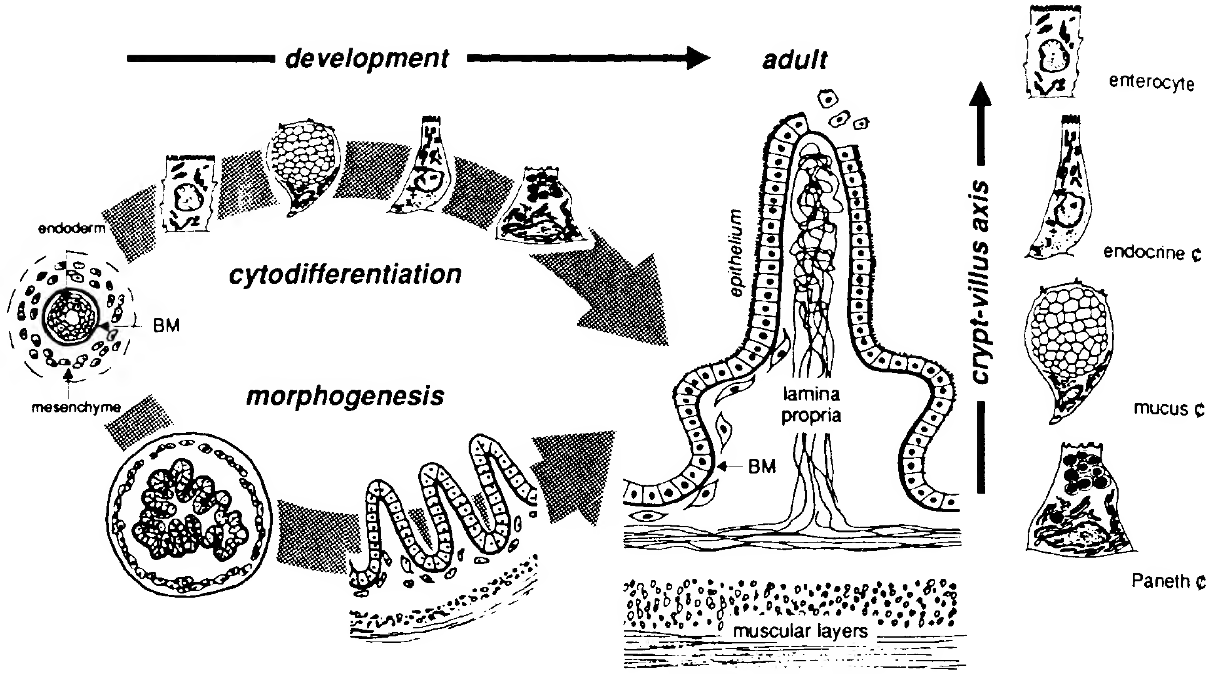

Figure 1. Schematic representation of the main morphological features and onset of the major epithelial cell types during intestinal morphogenesis and crypt to villus migration. The endoderm progressively differentiates into the villus epithelium; in the adult, cell diversification arises from the crypt compartment located at the base of the villi. The mesenchyme gives rise to the connective tissue - the lamina propria - and to the muscular layers. The subepithelial basement membrane (BM) is located at the epithelial/mesenchymal interface from early stages of development up to the adult stage. ¢: cell.

synchrony with epithelial cells strongly suggested that epithelial/mesenchymal interactions were still operating in the adult mature organ. This hypothesis has been demonstrated experimentally in recombination experiments showing that neonatal crypt cells as well as fibroblasts from the neonatal lamina propria retain properties similar to those of their embryonic forms[48,68].

It is well established that in multicellular organisms extracellular matrix (ECM) molecules control cell growth and differentiation This ECM network includes the interstitial matrix and basement membranes (BM). Interstitial matrix surrounds cells in the stromal connective tissue compartment, while BM are specialized sheet-like ECM that separate the connective tissue from epithelia, muscle fibers, blood vessels and nerves. Little is known about the functional role of ECM components in intestinal tissue although numerous sporadic data on location/expression of ECM molecules and receptors are available. The aim of the present review is 1) to integrate these descriptive data in order to understand how ECM could contribute to cell movements, and lead to the establishment and maintenance of polarized epithelial cells, and 2) to describe in vitro models that allow the study of the regulation of cell-ECM interactions.

Changes in ECM organization accompany morphogenesis of the developing intestine

ECM is formed by a complex set of collagens, non-collagenous glycoproteins and proteoglycans that has a unique composition in each organ. Expression of ECM molecules is tightly regulated; some are transiently expressed at particular times in development, whereas others are continually expressed up to adulthood.

At the electron-microscopic level, the subepithelial basement membrane corresponds to a continuous sheet of amorphous electron-dense material, also referred to as the 'basal lamina'. Electron-microscopic immunostaining of rat duodenum performed by Laurie et al.[77] showed for the first time that type IV collagen, laminin, and heparan sulfate proteoglycan were not layered, but were integrated together in the basal lamina. The distribution of known matrix components within the entire human and murine developing intestinal mucosa has been reported in comprehensive immunohistological studies[10,49,125,127]. The location of the major BM components in the adult intestine is schematically summarized on figure 2. All together, the data revealed that BM components are present at the intestinal epithelial/mesenchymal interface early in embryonic development and that changes in the spatial distribution of some ECM proteins are associated with morphogenetic processes.

Laminin is a glycoprotein having an approximate molecular weight of 900 kDa. This major component of the basement membrane is a cross-shaped heterotrimeric molecule. Laminin isolated from the Engelbreth-Holm-Swarm (EHS) tumor (laminin-1) is composed of three

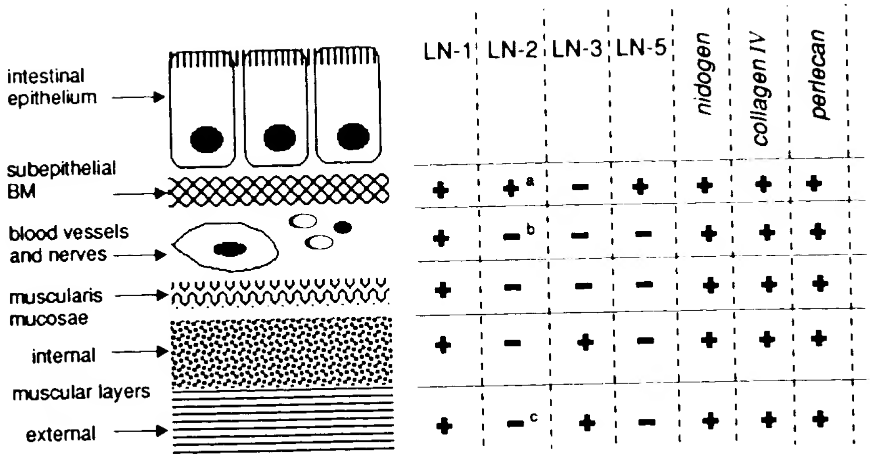

Figure 2. Summary of the distribution of the main basement membrane molecules as revealed by immunofluorescence in the different layers of the adult intestine. Presence (+) or absence (−) of fluorescence. LN-1, LN-2, LN-3 and LN-5 correspond respectively to EHS-laminin, merosin, S-laminin and nicein. Concerning LN-2, it should be pointed out that [a] the staining is restricted to the BM underlying the crypt region; in [b] labeling is found some fibers underneath the crypts and in [c] capillaries of the muscle.

peptidic chains A, B1 and B2[11] recently named $\alpha 1$, $\beta 1$ and $\gamma 1$[20]. Using polyclonal antibodies, laminin-1 was detected immunocytochemically at 12 days of gestation in the rat intestine[127] and at 8 weeks of gestation in the human[10] at the epithelial-mesenchymal junction as well as around a few cellular structures (presumably blood vessels) scattered within the mesenchyme. At a later developmental stage in rodent, just before villus formation, the staining observed in the mesenchyme was intensified and became confined to two distinct areas: the zone immediately beneath the epithelial-mesenchyme interface, and the most peripheral zone of the mesenchyme which will differentiate into muscular layers[127]. Throughout the following developmental period, laminin-1 was still detected at the boundary between epithelial cells and the stromal compartment; during this villus elongation period, the BM staining was however more regularly found at the base of the villi. In the adult, laminin-1 was linearly distributed from crypts to villus tips. By in situ hybridization, laminin $\gamma 1$ messenger RNA in the 12 day fetal mouse intestine was detected exclusively in the mesenchymal cells and preferentially in those immediately adjacent to the epithelium. Thereafter, laminin $\gamma 1$ mRNA expression was found to be strongest in cells forming the muscle layers, the muscularis mucosae and the lamina propria[119].

The study of neosynthesized laminin-1 from rat intestinal segments taken at various stages of development, purified by affinity chromatography on heparin-Sepharose has revealed interesting changes in the level and molecular forms of laminin-1 as a function of intestinal development[122]. Indeed, the maximal biosynthetic activity of laminin occurred in rat intestinal tissues during the gestational period (16–18 days) showing that the fetal intestine synthesizes very large amounts of laminin-1 at the same time as the commencement of intestinal differentiation, i.e. villus emergence and individualization of the smooth muscle layers proceed. Another peak of laminin-1 synthesis, although weaker than the first one, was detectable when crypts formed (zone of the dividing cells) by invagination of the base of the villi into the mucosalconnective tissue. Evaluation of relative proportions of individual laminin polypeptides showed that the intestine, like many organs, produced laminin $\beta 1$ and $\gamma 1$ subunit forms in excess of $\alpha 1$ subunit whatever the developmental stage considered. Interestingly, the ratio of the amounts of $\alpha 1$ and $\beta 1/\gamma 1$ polypeptides varied during morphogenesis, $\alpha 1$ chain level being maximal during villus formation[71,122]. Northern blot analyses partly confirmed these observations; indeed, examination of RNA transcripts for the laminin $\beta 1$ and $\alpha 1$ chains in the developing rat intestine revealed peculiar temporal patterns (fig. 3). Amounts of steady-state $\beta 1$ and $\alpha 1$ mRNA expression were much higher in the fetal intestine compared to the adult mature organ. Surprisingly, the laminin $\alpha 1$ chain probe hybridized to a ~ 6 kb band and not to the predicted 9.5 kb transcript (fig. 3). We are uncertain about the significance of this small transcript. Yet Vanden Heuvel and Abrahamson[148] recently showed that both 9.5 kb and 6 kb transcripts exist in developing kidney, and that this smaller transcript is enriched in tubular fractions. However, Klein et al.[71] could identify a very weak 10 kb signal for laminin $\alpha 1$ mRNA in total RNA from 13-day-old embryonic intestine but not from later stages. Furthermore, using three different cDNA fragments detecting different regions of laminin $\alpha 1$ chain mRNA, they were able to identify a 10 kb signal from poly(A) + RNA; according to this study, no truncated forms of $\alpha 1$ chain mRNA were expressed in any tissues analyzed at this embryonic stage.

Despite a high level of cell migration and turnover in the adult mature intestine (2 to 6 days ~~according~~ to the species), production of new BM is low. Indeed, laminin

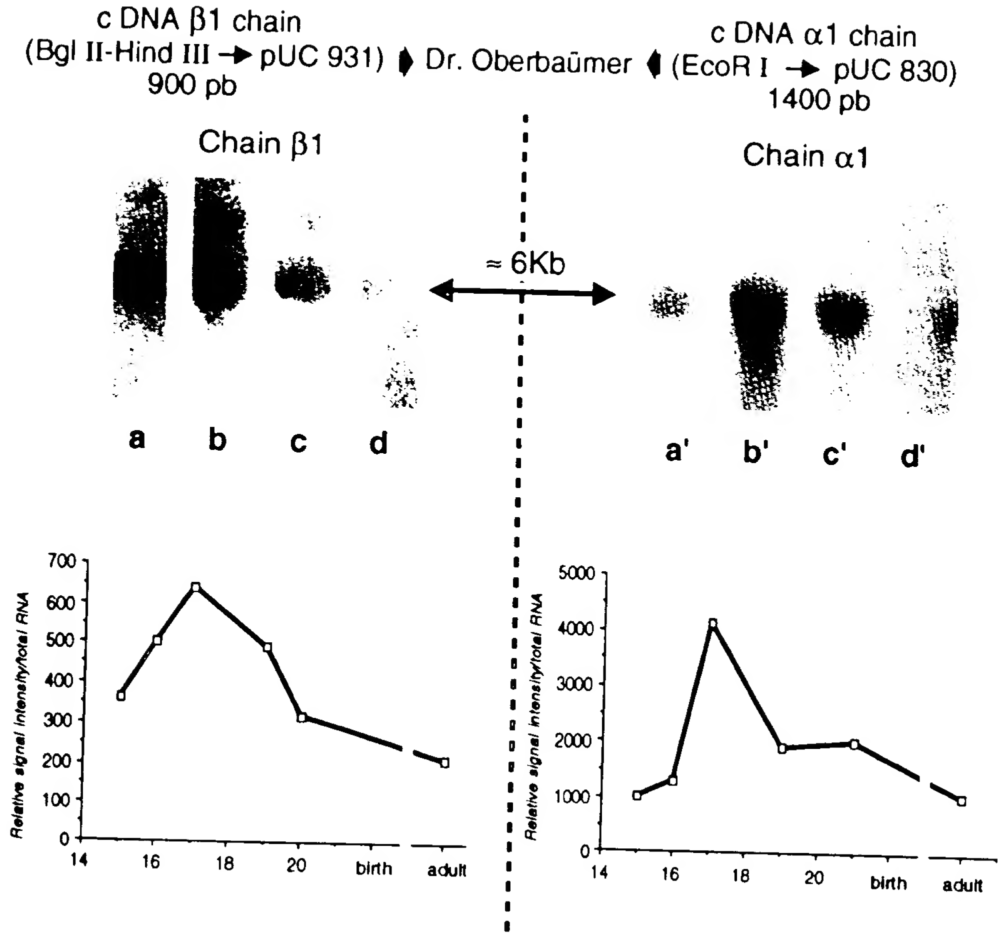

Figure 3. Northern blot analysis of mRNA for laminin $\beta 1$ (a–d) and laminin $\alpha 1$ (a'–d') chains in RNA isolated from 15-(a, a'), 17-(b, b'), 19-(c, c') day-old fetal rat intestines and from adult rat intestines (d, d') (Simo et al. unpubl. results). Integrity of RNA from each preparation was determined using an actin probe. The lower part of the figure depicts semi-quantitative profiles of laminin $\beta 1$ and $\alpha 1$ mRNAs in the developing intestines. RNAs were electrophoresed and transferred onto nitrocellulose filters, and hybridized with the corresponding cDNA probes kindly provided by Dr Oberbäumer[34,96]. The cDNAs were ^{32}P-labeled using the random priming technique; specific activity was between 10^7–10^8 cpm/μg of DNA.

messenger RNA assessed by in situ hybridization[119] and Northern blot (fig. 3) is far lower in the adult than during fetal stages. Moreover, Trier et al.[144] clearly demonstrated by labeling laminin molecules in vivo with anti-laminin IgG that laminin-1 turnover occurs focally in the BM of adult mouse jejunum over weeks; the persistence of some staining along the length of the crypt-villus axis for as long as six weeks provides strong evidence that the BM does not comigrate with its overlying epithelium or underlying myofibroblasts. Immunohistological studies using anti-laminin-1 polyclonal antibodies did not reveal any clear gradient along the crypt-villus axis in adult rodent or human intestine[9,126,127] (fig. 4*A*). Yet, in the adult rodent organ, laminin $\beta 1/\gamma 1$ chains were found homogeneously distributed in the crypt and villus basement membrane, whereas $\alpha 1$ chains were restricted to the crypt zone[127]. Interestingly, in the adult human intestine the $\alpha 1$ chain, found at the subepithelial basement membrane, presented a decreasing gradient of intensity from the tip of the villus to the crypt mouth; no obvious staining was observed in the crypt-cell compartment (fig. 4*B*). These observations point to the potential expression of laminin isoforms along the crypt-villus basement membrane.

From recent studies, it has become apparent that laminin-1 is a member of a family of proteins[20,145]. In fact, molecular biology techniques and availability of specific antibodies have demonstrated 5 laminin variants up to now. These members of the laminin family are expressed in different tissues and at different times during development. An $\alpha 1$ chain variant, the $\alpha 2$ chain (formerly called M chain), has been characterized and shown to be associated with the classically described $\beta 1$ and $\gamma 1$ chains forming the laminin-2 or merosin molecule[41,80]. Laminin-2 was first identified as a protein present in basement membranes of trophoblasts, Schwann cells, and striated muscle[80]. The use of monoclonals against the high molecular weight $\alpha 2$ chain emphasized the heterogeneous composition of laminin

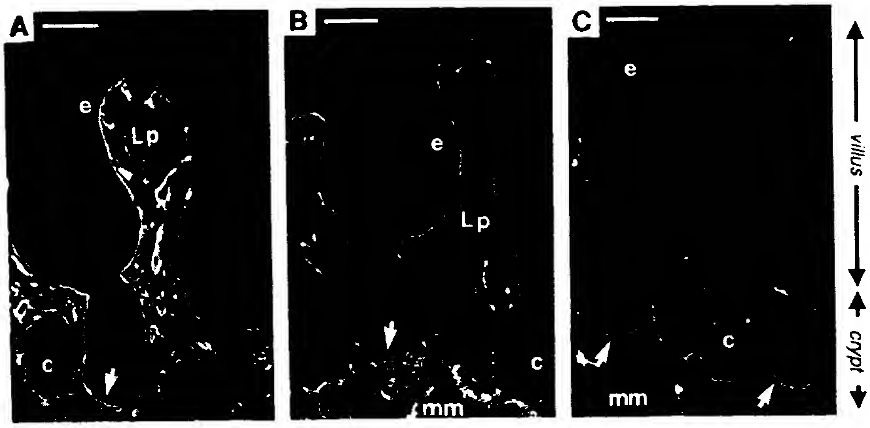

Figure 4. Immunostaining showing the location of *A* laminin-1 detected with a polyclonal antibody, of *B* laminin $\alpha 1$ chain with mAb 4C7, and of *C* laminin $\alpha 2$ chain with mAb 4G9, on cryosections of intestine from a 4 year-old child. e, epithelium; Lp, lamina propria; mm, muscularis mucosae; c, crypts; arrows point to the epithelial stromal interface in the crypt region. Bar: 50 µm.

molecules in the adult human intestine. $\alpha 2$ chain was exclusively present in the basement membrane at the bottom of the crypts[9,126] (fig. 4C) thus depicting a complementary pattern of $\alpha 1$ and $\alpha 2$ chain expression in the human intestine. These data show that at least in the mature human intestine, $\alpha 1$ and $\alpha 2$ chains are mutually exclusive. It is worth noting that $\alpha 2$ chain was only expressed when crypt downgrowths occurred, exhibiting immediately a typical localization in the crypt region[126].

The family of laminin variants also includes a 600 kDa molecular weight glycoprotein termed BM-600, kalinin or nicein[85]. This protein, now called laminin-5, was successfully immunoprecipitated from cultures of keratinocytes from healthy donors but not from patients suffering from a lethal dermatosis, Herlitz junctional epidermolysis[150]. In addition, this molecule has been shown to immunolocalize to anchoring filaments bridging hemidesmosomal structures to lamina densa[108]. Taken together, these data indicate a possible role for laminin-5 in cell-substrate adhesion and BM cohesion. In the small intestine, indirect immunofluorescence studies revealed that, in opposite to laminin-1, laminin-5 was restricted to the subepithelial BM; BM of blood vessels and nerves were not stained (Orian-Rousseau et al., unpubl. data). Yet, a clear increasing gradient of staining was obvious from the mouth of the crypt gland to the villus tip; the staining appeared scarcely if at all detectable in the crypt region. This distribution pattern of laminin-5 was superimposable on one hand to that of HD1 (Fontao et al., unpubl. data), one of the intracellular hemidesmosomal proteins mediating cell adhesion to the extracellular matrix[53] and on the other hand to the $\beta 4$ integrin subunit[9,126]. The $\alpha 6\beta 4$ integrin heterodimer is known to be localized in hemidesmosomal plaques in cornea and skin[59,136]. However, these hemidesmosomal structures are known to be associated with collagen VII, a major structural component of anchoring fibrils[112] that is absent from the intestine[79]. Thus, it is interesting to note that a certain number of the hemidesmosomal components are mostly located at the basal surface of differentiated cells that migrate up the villi; this suggests that in contrast to the classically described hemidesmosomes, the 'hemidesmosome-like structures' found in the intestine do not function as an anchorage device.

The other laminin variants, S-laminin (laminin-3), S-merosin (laminin-4) and K-laminin (laminin-6) have not so far been studied in the intestinal organ. However, in a comprehensive study dealing with the expression of laminin variants in the adult human smooth muscle, Glukhova et al.[45] found that S chain, newly called $\beta 2$ chain, could be detected only in the adult muscular layers. This is in contrast with laminin-1 which is the predominant laminin variant found in the developing human colon and with laminin-2 which is restricted to capillaries in the adult muscle coat. Generally, the nature of the effects of each laminin variant on cell behavior (migration, proliferation, differentiation) remains largely unsolved and is beginning to be analyzed mainly by in vitro model systems.

Type IV collagen, another major structural component of BM, is a triple-helical molecule composed of three α chains. Collagen IV molecules which were assumed to contain two $\alpha 1$ (IV) chains and one $\alpha 2$ (IV) chain are now known to harbor also $\alpha 3$ (IV), $\alpha 4$ (IV), $\alpha 5$ (IV) and $\alpha 6$ (IV) chains[98,147]. To date, five genetically distinct type IV isoforms have been described in mammals[158]. The existence of numerous α chains of collagen IV that can be connected in various arrangements strengthens the complexity of the BM organization. In the adult and developing gut, the expression of subepithelial collagen IV molecules strictly paralleled that of laminin-1[10,127], that is homogenously distributed as development proceeds along the mature crypt-villus axis. In situ hybridization allowed the demonstration of the widespread

presence of α1 (IV) collagen messenger in the intestinal embryonic mesenchyme and in the differentiated mesenchymally-derived compartments (lamina propria, muscular cells . . .). Redistribution of type IV mRNA in the mesenchyme, leading to its confinement just beneath the epithelial mesenchymal interface, paralleled the segregation of type IV collagen antigens. During villus outgrowth, high levels of mRNA were seen in the protruding villi suggesting that new BM material has to be laid down. Finally, as for laminin in the mature adult organ, no type IV mRNA could be reliably demonstrated by in situ hybridization[126]. Similar data have been produced by Weiser et al.[153], who showed by dot-blot hybridization analyses that there was little, if any, evidence for the presence of the transcripts for collagen α1 (IV) and α2 (IV) chains in the epithelial cells. Furthermore Northern blot hybridization analysis of rat intestinal poly(A)$^+$RNA showed that slight signals were observed and restricted to the lamina propria fraction. Recently, α5 (IV) and α6 (IV) mRNA transcripts of ~7 kb were shown to be expressed in a 24-week human fetal intestine although in low quantities. The fact that a deletion in α6 (IV) collagen gene occurs in inherited smooth muscle tumors, leads to the assumption that these molecules could be critical for normal smooth muscle differentiation[159].

Perlecan. Basement membranes also contain perlecan, a large low-density proteoglycan (PG) composed of heparan-sulfate (HS) side chains which was first isolated and characterized[51] from the EHS tumor. Although heparan sulfate proteoglycans are important ECM components that appear to have multiple functions, precise studies concerning their expression in the intestine are quite rare. Polyclonal antibodies prepared against EHS perlecan and directed against the core protein of the proteoglycan were used to examine the distribution of this BM component in the mature rat intestine and during its morphogenesis[125]. Again, the overall distribution of perlecan is similar to that described previously for the other BM components, laminin-1 and type IV collagen: prominent staining at the subepithelial BM and around muscular cells. However, contrasting with the regular deposition of the latter molecules at all stages of development, changes in the staining pattern of perlecan were observed around birth in the rat intestine; indeed at this period, the labeling of the BM became discontinuous and irregular from the middle to the tip of the villi. Similar transient microheterogeneities in the deposition of BM molecules have been described in other organs undergoing morphogenetic movements (for review see ref. 143). Anti-liver HSPG IgG as well as antibodies raised against HSPG purified from PYS-2 cell cultures were also able to recognize the intestinal subepithelial BM[30,134]. Yet, the PYS-2 HSPG antibodies did not label most smooth muscle tested including that of small intestine[30]. Differences in the size of the HSPG core proteins have been found and recent data provide evidence that minor variants of perlecan appear to be generated by alternative splicing[97]. These data point to structural variations between HSPG molecules in various types of basement membranes, which may account for differences in their overall function.

Entactin/nidogen. Other molecules have been described as associated with the BM. Among them entactin, also called nidogen, is a sulfated multidomain glycoprotein of 150 kDa. Its structure and biological properties have been reviewed recently[25]. Increasing evidence suggests that entactin/nidogen plays an essential role in the assembly of basement membranes forming a link between laminin-1 and type IV networks[5]. Similarly, nidogen mediates the formation of a ternary complex between laminin-1 and the core protein of proteoglycans[5]. Therefore, one can understand that the pattern of expression of entactin/nidogen strictly follows that of other BM molecules[127]. Electron immunohistochemistry revealed that entactin/nidogen was found almost exclusively in the lamina densa[87]. By Northern blot analysis, a significant amount of nidogen mRNA was found in a 12.5 gestational day mouse intestine, the only stage studied[140].

SPARC/BM40 and chondroitin sulfate proteoglycan. Other BM components have been detected in the intestinal subepithelial basements. They concern SPARC/BM40 and chondroitin sulfate proteoglycan (CSPG). SPARC is often considered as a basement membrane protein since it is produced in large amounts by EHS tumor[39]. It has a broad tissue distribution (for review see ref. 98). It is worth noting that in immunohistochemistry performed on various fetal and adult mouse organs, its preferential location was in epithelia exhibiting high rates of turnover such as the gut[111]. Production of core protein-specific monoclonal antibodies that recognize a large high density proteoglycan (bearing 13–22 chondroitin sulfate glycosaminoglycan chains) allowed McCarthy and Couchman[90] to conclude that one CSPG is a constituent of most basement membranes. The subepithelial BM in the gut, as well as basement membranes surrounding smooth muscle cells and blood vessels, were recognized by these antibodies.

Fibronectin and type III procollagen. All the other matrix molecules found in the intestinal lamina propria and smooth muscle layers belong to the family of interstitial molecules. Concerning fibronectin, several studies have localized this glycoprotein to the basement membrane of a variety of tissues (for references see ref. 75). It should be noted that at early stages of development (12 days in the fetal rat), immunostaining of fibronectin was clearly detected as a linear band at the endodermal mesenchymal interface (unpubl. data). These data are in accordance with those of Laurie et al.[77] showing by electron microscopic immunostaining that type IV col-

lagen, laminin-1, heparan sulfate proteoglycan and fibronectin did not occur in separate layers but were integrated into a common structure. Later on, during formation of villi (17 days of gestation in the rat), fibronectin as well as type III procollagen disappeared from the top of the protruding connective tissue[127]. Finally, in the adult rodent and human intestines, fibronectin exhibited a decreasing gradient of intensity from crypt region towards the top of the villus core[10,108,127]. In the smooth muscle layer of mouse small intestine, fibronectin was localized abundantly in the narrow space between smooth muscle cells[75].

Tenascin. The first demonstration of the presence of tenascin (also known as cytotactin and J1) in the intestine was published by Thor et al.[141]. Tenascin is a hexameric glycoprotein with disulphide-linked subunits originally described as myotendinous antigen[22]. This molecule is expressed at the boundary between epithelial cells and the lamina propria in an increasing gradient towards the villus tip in mouse[4,141], rat[128] and human adult intestine[7]. More precisely, tenascin was expressed in association with ECM surrounding subepithelial fibroblasts of the lamina propria[102]. The fact that fibronectin and tenascin display inverse gradients in immunofluorescence intensities led the latter authors to speculate that this shift may trigger the cell shedding process. Further arguments were brought out by experiments using colonic cancer epithelial cells (HT29 cells) that adhered to fibronectin, but not to tenascin[102]. In addition, tenascin reduced the adhesion of the cells to fibronectin[23,102]. During development, the first onset of tenascin was only obvious in the intestine at day 14 in the mouse embryo; at this stage a faint labeling occurred mostly in the peripheral part of the mesenchyme corresponding to the region that will develop into outer muscle layers. From the 17th day of gestation, the increasing gradient from the crypt base to the top of the villus was established, becoming more accentuated in the adult organ[4,103]. A similar expression pattern of tenascin was obvious in the developing human intestine[8]. It is worth noting that the molecular form of tenascin changes as development proceeds[4,8]. When first detected, a 210 kDa chain was strongly expressed, but at birth the relative amount of 260 kDa chain increased becoming more pronounced in the adult intestine. These two tenascin polypeptides arise through an alternative RNA splicing; a 6 kb mRNA predominated during embryogenesis whereas the 8 kb mRNA appeared later[154], contrary to the situation found in kidney.

Collagen. New analytical tools have led to the discovery of the impressive diversity of the collagen family (for review see ref. 147). At present, only sporadic data are available for the intestine, compared to the wide array of these molecules. Among them, type I collagen has been found to be the major collagen type in the adult and developing human small intestine and colon[47,89] followed by type III collagen[124]. High levels of the corresponding mRNAs were mainly observed in the submucosa; no staining occurred in the muscular layers[115]. Although collagen II has traditionally been considered specific for cartilage, the intestinal tissue has been found to contain small amounts of its $\alpha 1$ (II) mRNA; in addition, $\alpha 2$ (XI) collagen seems to be synthesized in this organ, although the signal intensity in the RNAase protection assay was lower than that obtained with collagen II probes[114]. By calculating the relative amounts of various α chains, Graham et al.[47] showed that collagen type V, composed of $\alpha 1$ (V), $\alpha 2$ (V) and to a less extent of $\alpha 3$ (V), constituted a very significant proportion of total collagen in the intestine as compared with other tissues. Cells expressing high amounts of $\alpha 1$ (XIII) collagen mRNA were identified in the mucosal layer of the colon and the small intestine[115]. Various cultured cell lines, including human colonic adenocarcinoma cells are able to express multiple mRNA variants of $\alpha 1$ (XIII) chain[61]. Finally, immunohistochemical studies demonstrate that type XIV collagen is present around smooth muscle cells[21]. Conversely, no type VII collagen or type VIII collagen were expressed in the intestine[79,91,112].

Proteoglycans, large proteins carrying sulfated polysaccharides (glycosaminoglycans: GAGs) are present in virtually all mammalian tissues. The diverse structures of proteoglycans found in different tissue locations reflect their unique biological properties[142,156]. Quantitative as well as qualitative follow up of the GAGs have been performed in the developing gut[16,56,113]. Chemical analysis of purified GAGs revealed that all classical molecules are found in the intestine: hyaluronic acid (HA), heparan sulfate (HS), dermatan sulfate (DS) and chondroitin 4 and 6 sulfate (CS4 and 6). The overall GAG synthesis was maximal during fetal life and declined dramatically after birth, reaching relatively steady values maintained until adult stages[16]. One major alteration in the evolution pattern of the individual GAG species concerns the important drop in HA molecules, which accounts for the overall GAG decrease. Moreover, poorly or even completely unsulfated HS molecules were produced by early fetal intestines. Histochemically, GAGs occurred predominantly in the loose connective tissue of the villi[44,56]. A DS-PG was found located in the same region as well as in the muscle layers[131]. Among leucine-rich interstitial proteoglycans is found lumican, which contains several keratan sulfate chains. This proteoglycan showed a limited distribution in connective tissue, and was expressed in cornea as well as in muscle and intestine[12]. In the intestine, as in other tissues, the cDNA clone to lumican hybridized to a 2.0 kb mRNA, and the molecule was synthesized as a precursor protein. Its functional role remains to be elucidated, although it may regulate collagen fibril assembly through its core protein

Thrombospondin 3. Finally, thrombospondin 3 (TSP3), belonging to a growing family of cell surface and extracellular matrix molecules, has been found to be expressed in the intestine by in situ hybridization. Its pattern of expression in various organs led to the hypothesis that TSP3 could participate in specifying or maintaining mesenchyme differentiation into muscle, bone and cartilage[104].

The increasing interest in the field of the extracellular microenvironment has led to the finding of a huge diversity in the molecular composition of the ECM. Diversity begins at the DNA level as examplified by molecules composed of chains expressed from distinct genes often distributed on different chromosomes. The second level of diversity arises from the alternative splicing of several genes resulting in different polypeptide chains. In addition, some molecules can be differentially expressed as isoforms (as laminin, type IV collagen) varying in their constituent chains or their glycosylation pattern. As an example, the $\beta 1$ chain of EHS laminin participates in the trimeric association in laminin-2 and laminin-6. Variations in the relative ratios among the major well-defined components, and in the expression of minor molecules further increase the structural organ-specific and spatio-temporal ECM heterogeneity. Finally, the orientation and assembly of the various molecules in a given tissue will direct precise cellular functions (for a review see ref. 98). In the case of the intestinal tissue, it is tempting to speculate that ECM and particularly BM microheterogeneities and asynchronous expression of their constituents during intestinal development and along the crypt-villus axis in the mature organ, may account for the functional diversity of the extracellular microenvironment.

Expression of receptors could contribute to cell movements during morphogenesis and in the adult

The differential expression and functional state of receptors for BM molecules may also contribute to morphogenetic movements and differentiation of epithelial cells during development and cell renewal. A large number of cellular receptors able to bind ECM molecules has been described[55,109,130].

Integrins appear to be the major receptors by which cells attach to the ECM. Integrins are a large family of at least 21 different heterodimers, each consisting of an α- and β-subunit that associate nonconvalently. It is now known that at least five α-subunits – $\alpha 1$, $\alpha 2$, $\alpha 3$, $\alpha 6$, and $\alpha 7$ – can combine with the $\beta 1$-subunit to form heterodimers able to bind to EHS laminin[55]. In addition, $\alpha 6\beta 4$ as well as $\alpha 6\beta 3$ have been reported to function as laminin receptors in several organs and in various cell cultures[78].

Lamin receptors. The most prominent laminin receptors found in the developing intestine and in the adult organ in several species (human, mouse, chicken) are $\alpha 6\beta 1$ and $\alpha 6\beta 4$. These have been found located underlying the epithelium in various segments of the gastrointestinal tract[6,9,18,24,64,73,92,126,133]. The developmental expression of $\alpha 6$ and $\beta 4$ subunits was not coordinated in two species studied (human, mouse), the $\alpha 6$ expression preceding that of $\beta 4$[126]. The fact that the $\beta 4$ subunit was not detected at early stages of morphogenesis when laminin-1 is present, indicates that the $\beta 4$-containing integrins are not involved in the binding of embryonic epithelial cells to BM laminin. Concerning the expression of $\alpha 6$, $\beta 1$ and $\beta 4$ along the crypt-villus axis in the adult organ, it should be pointed out that 1) in the human intestine, all three subunits were expressed at the base of all enterocytes from the base of the crypt to the tip of the villus, while in contrast 2) in the mouse intestine, $\alpha 6$ staining was mainly confined to the crypt cell compartment unlike $\beta 4$ staining which was regularly observed along the crypt-villus axis[9,126]. The differential immunodetection of $\alpha 6$ and $\beta 4$ subunits along the crypt-villus axis in the mouse intestine is intringuing. However, these findings concerning the restricted distribution of the $\alpha 6$ subunit are in accordance with the location of laminin $\alpha 1$ chain, known to have a C-terminal sequence which binds to $\alpha 6\beta 1$ integrins[132]. One can therefore postulate that differentiation occurring along the crypt-villus axis is not only associated with changes in integrin expression but can also be linked to possible changes in receptor conformation, so that the antibody is no longer capable of recognizing it[54]. Furthermore, it has been shown that mouse $\alpha 6$ subunit can exist in two versions, $\alpha 6A$ and $\alpha 6B$, which contain structurally distinct cytoplasmic domains; recent findings indicate that many differentiated cell types express $\alpha 6A$[29]. Therefore, one can also postulate that these two $\alpha 6$ isoforms bearing distinct cytoplasmic domains are differentially expressed along the crypt-villus axis in the mouse intestine and/or differ in their affinity for laminin. Integrin subunits $\alpha 1$ and $\alpha 2$ were located more or less regularly along the villus, while $\alpha 3$ subunit was expressed mostly by enterocytes lining the upper villus (refs 6, 24, 73 and authors' unpubl. data). In addition, some evident immunofluorescence staining localized in cell-cell adhesion contacts within the epithelium is even more obvious in the colon compared to the small intestine (ref. 160 and authors' unpubl. data). The new isoform of the laminin integrin, $\alpha 7\beta 1$, was expressed exclusively in the intestinal muscular layers[27].

Receptor binding fibronectin. The $\alpha 5\beta 1$ receptor binding fibronectin has not been detected[24] or is barely detectable[6] at the base of intestinal epithelial cells. Nevertheless, $\alpha v\beta 6$, another fibronectin-binding heterodimer, has been shown to be expressed in small intestinal and colonic epithelium[17]. Finally, the $\alpha 8\beta 1$ integrin, whose ligand is at present unknown, is moderately expressed in the epithelial cell layer[13].

Hyaluronate receptor and non-integrin receptor. There are additional receptors for ECM in the intestine that are worthy of note. The first subset concerns CD44 which is the major cell surface receptor for hyaluronate[3]. This integral membrane glycoprotein was prominently expressed in the BM regions lining exclusively the base of the crypts, corresponding to those regions where proliferation of epithelial cells occurs[2]. More generally, this receptor has been found preferentially expressed on epithelial cells undergoing active cell division[2]. The second subset is a 67 kDa protein, a non-integrin receptor, with high affinity for laminin[130]. Recent data of Rao et al.[106] showed that the 67 kDa mRNA levels were about ten times greater in crypt compared to villus cells in the adult intestine. The fact that the 67 kDa is a common feature of mitotically active cells is corroborated by the fact that its expression was increased in a variety of human adenocarcinomas[26]. Although this 67 kDa laminin receptor is still the subject of controversy, its role in intestinal physiology in the light of present data seems to be rather important.

Dual origin of the subepithelial basement membrane in the developing intestine

New analytical tools and models allowed more recent studies of tissue interactions and BM formation. Deposition and assembly of defined molecules into a basement membrane result from complex mechanisms which are probably unique for each system and depend on the developmental fate, physiological state of the system and environmental conditions[50]. It is now largely accepted that cooperation between various cell types is necessary for BM deposition.

The classic concept of an exclusive epithelial origin of the BM has been revisited since 1977. Indeed, Lipton et al.[82], using cultures of embryonic quail myoblasts, were probably the first to provide evidence for a dual origin of the BM showing the contribution of fibroblasts. Various experimental techniques are currently used to study the expression of basement membrane molecules. They include immunohistochemistry, biochemical approaches, or detection of transcripts by in situ hybridization on isolated tissue compartments or cell lines. Apart from the minor limitations of each model (such as cellular contamination in the case of isolated epithelial or mesenchymal cell preparations, abnormal cell behavior or loss of differentiation of cultured cells, threshold sensitivity), they all have a major drawback. Indeed, the fact that a tissue compartment expresses a given basement membrane molecule does not necessarily imply that this molecule is deposited at the BM region. Autoradiographic studies, which circumvent this problem, unfortunately do not allow discrimination between individual components. The strategy designed by Sariola et al.[illegible], that is interspecies hybrid glomeruli combined with species-specific antibodies, deserves special attention. The major advantage of this model is that it allows us to distinguish the deposition at the basement membrane level of a single molecule.

To study the tissue origin of BM in the gut we performed similar experiments using recombinants between chick and rodent intestines. As depicted in figure 5, isolation of pure tissue compartments was achieved with embryonic chick intestine and with fetal rat or mouse intestines, and interspecies recombinants were performed. After growth of the grafted implants, species-specific antibodies were applied on cryosections of the developed intestinal hybrid structures. This strategy allowed us to conclude that the intestinal subepithelial BM is composed of molecules produced by both cell populations. This conclusion strengthens the finding that heterotypic cell cooperation is necessary for the formation of a structured BM.

Type IV collagen. We could show that mesenchymal cells are the principal endogeneous source of this molecule[123]. This conclusion is confirmed by the localization of type IV collagen mRNA in the mesenchyme or mesenchyme-derived cellular elements of the lamina propria[124]. Yet, regional differences in basement membrane synthesis and assembly seem to occur, since in the stomach of a 12.5-day mouse embryo the collagen IV transcripts were detected in both epithelium and mesenchyme[140]. The data of Weiser et al.[153] concerning the adult mature organ are in accordance with a mesenchymal origin of collagen IV. These authors clearly showed that the mRNAs for $\alpha1$ (IV) and $\alpha2$ (IV) collagen were most abundant in the lamina propria; no collagen IV mRNA were evident in the enterocyte fractions even in oligo (dT)-selected RNA. It has to be noted that, during the reestablishment of the BM in the interspecies reassociations, some type IV collagen can be deposited, although transiently, by the epithelial cells[129]. The surprising but interesting finding of mesenchymal origin of collagen IV is not restricted to the intestinal system. In particular, Marinkovich et al.[84] provide evidence that dermal fibroblasts synthesize and deposit type IV collagen, as well as type VII collagen and laminin, into the basement membrane zone; these authors used as a strategy dual species cultures of bovine keratinocytes and human fibroblasts analyzed by immunofluorescent microscopy with human specific antibodies against BM components.

Perlecan. Unlike collagen IV, HS-PG (or perlecan) deposition at the BM level is achieved by the epithelial compartment; in this case HSPG labeling was exclusively with the antibodies that react specifically with the species from which the epithelial cells were taken[125].

Laminin. Concerning laminin, the third class of BM molecule analyzed, it should be noted that the laminin $\gamma1$ chain transcripts have been localized to the mes-

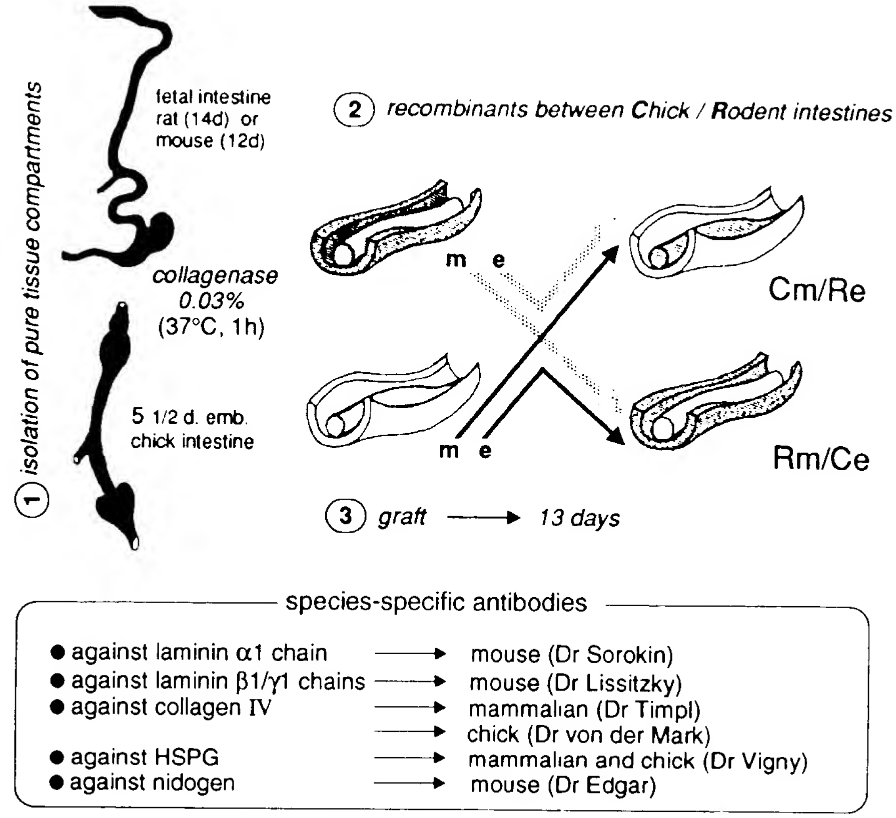

Figure 5. Schematic representation of the experimental procedure used for the study of the cellular origin of BM in the intestine. *1* Isolation of pure tissue compartments from the intestine is performed as follow: 12-day-old or 14-day-old fetal mouse or rat intestines, $5\frac{1}{2}$-day-old embryonic chick intestine are dissected out and incubated in a collagenase solution (1h, 37 C) to disrupt the BM; *2* after opening the intestinal tube with iris scissors, the endoderm can easily be separated from the mesenchyme, and interspecies recombinants (Cm/Re: chick mesenchyme/rodent (rat or mouse) endoderm; Rm/Ce: rodent mesenchyme/chick endoderm) are performed; *3* they are then grafted into the coelomic cavity of 3-day old chick embryos or under the kidney capsule of adult nude mice to allow the development of the implants up to 13 days. The developed hybrid intestinal segments are taken at various periods of times. The species-specific antibodies recognizing either rodent or chick intestines are applied on cryosections performed through the hybrid intestine.

enchyme of embryonic intestine and in the lamina propria[119]. Yet by the filter hybridization method, messenger mRNAs for laminin $\beta 1$ and $\gamma 1$ chains were found in addition in the epithelial cell fraction in the adult[153]. From the analysis of the interspecies rodent/chick intestines with polyclonal or monoclonal antibodies recognizing rodent but not chick laminin, it can be conclude that laminin-1 has a dual, epithelial as well as mesenchymal, cellular origin[120]. Epithelial cells produce the three constituent chains of laminin during the whole development of the hybrids. In contrast, a precise chronology in the deposition of laminin chains by the mesenchyme could be seen: $\beta 1/\gamma 1$ chains were produced over the whole developmental period, while $\alpha 1$ chain deposition was delayed; this late expression of $\alpha 1$ chain may indicate that inductive influences emanating from the epithelial cells had occurred. Related to these data, expression of laminin $\alpha 1$ transcripts in mesenchymal cells localized immediately beneath the epithelium was found in organs such as gut and lung in the mouse embryo[140]. The asynchrony in the deposition of the constituent chains of laminin can be related to the data reported in the kidney: in this organ, $\beta 1/\gamma 1$ chains were constitutively expressed whereas the onset of $\alpha 1$ chain expression correlated with initiation of kidney tubules morphogenesis[72]. Immunoprecipitation of metabolically labeled molecules produced by the undifferentiated intestinal mesenchyme revealed only $\beta 1/\gamma 1$ chains of laminin[70] that could confirm the hypothesis of an inductive cell interaction process for synthesis of 1 chains; in similar experiments in the isolated endoderm, laminin $\alpha 1$ chain was coprecipitated with $\beta 1/\gamma 1$ chains. As the intestinal tissue is able to produce laminin variants (see above), further studies are necessary to determine their pattern of deposition in the BM. The possibility remains that each individual laminin chain can be secreted independently and be assembled according to a temporo-spatial pattern.

Nidogen/entactin. Found at the subepithelial intestinal BM, this has been shown to be a mesenchymal product (fig. 6) like in other tissues analyzed by in situ hybridization[35,140]. The crucial role of nidogen as an inter-

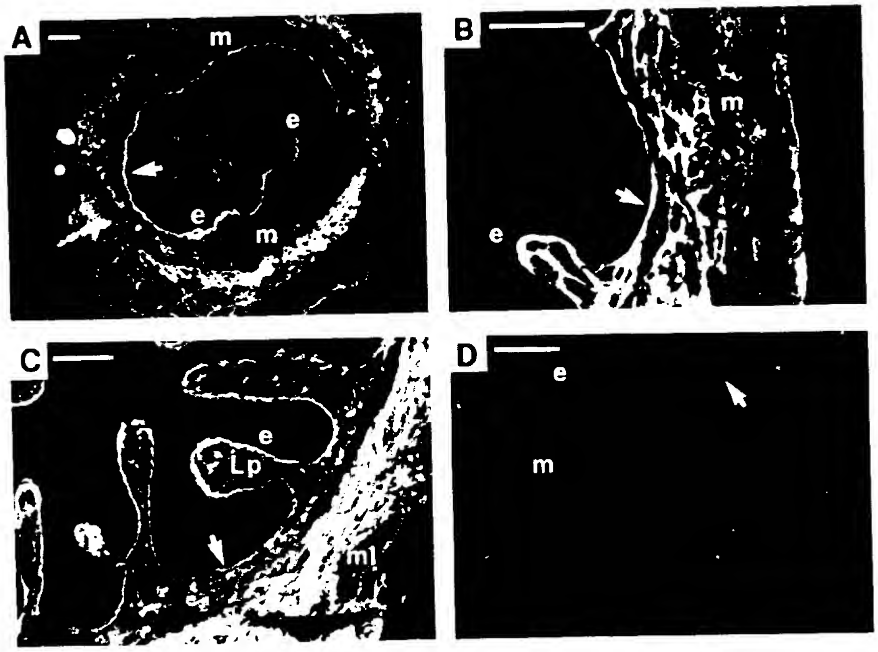

Figure 6. Cellular origin of nidogen (Simon-Assmann et al., unpubl. data). Immunodetection of nidogen molecules with rat monoclonal antibodies[38] recognizing specifically mouse but not chick antigens, on mouse mesenchyme/chick endoderm (A–C) and on chick mesenchyme/mouse endoderm (D) hydrid intestines at 4 (A), 6 (D), 10 (B) and 13 (C) days of development. e, endoderm or epithelium; m, mesenchyme; Lp, lamina propria; ml, muscular layers. Arrows point to the epithelial/stromal interface. Bars: 50 μm.

molecular linker, and of laminin α1 chain shown to be necessary for the formation of BM by MDCK epithelial cells in culture[40], leads to the speculation that both polypeptides play a key role in the formation of a stable basement membrane.

Models allowing the study of mechanisms of the role of ECM molecules in heterologous cell-cell interactions

Attempts to investigate the modulation of tissue-specific phenotypes in isolated epithelial cells have failed, mainly due to a rapid loss of cell function in culture[64]. These observations strengthen the idea that survival and differentiation of specific epithelial cells requires a precise molecular microenvironment provided by direct contact with living fibroblasts as in vivo. Indeed, a coculture system has been show to allow enterocytic differentiation starting from embryonic epithelial cells[66]. Endodermal microexplants were seeded onto a confluent feeder layer of fetal intestinal mesenchymal cells or skin fibroblasts. Whatever the cellular substrate, endodermal cells grew and, after about 4 days, covered the fibroblastic cell layer. Elaboration of complete BM comprising type IV collagen, laminin-1, nidogen and perlecan was obvious. The deposition of the BM molecules at the endodermal/fibroblastic interface was progressive and preceded the expression of epithelial differentiation markers, such as lactase. These data have been confirmed by Stallmach et al.[135] and Hahn et al.[49]. However, the latter authors found contrasting behavior of gastric versus intestinal mesenchyme for support of epithelial differentiation in vitro, emphasizing the concept of some regional specificity of the mesenchyme along the intestinal tube (see also Duluc et al.[36]). The induction of epithelial differentiation depends on the contiguity of vital mesenchymal cells, since fibroblast-derived matrices or irradiated fibroblasts were ineffective[66]. In addition, polarization of epithelial cells was not induced by any single type of matrix molecule tested (type I or IV collagen, fibronectin, laminin-1). By contrast, the use of EHS extract to which type I collagen has been added as a substratum led to a burst of cell differentiation accompanied by the expression of digestive enzymes; nevertheless, the survival time of these microexplants was rather limited as compared to coculture conditions[63]. The lack of clear effect such as differentiation when epithelial cells are cultured on isolated matrix molecules seems to be peculiar to the intestinal cells. Indeed, the morphology, behavior and even function of other cell types such as Sertoli cells or mammary epithelial cells in culture are greatly influenced by the extracellular matrix (for reviews see ref. 37, 138). It has clearly been shown that culture of epithelial Sertoli cells on laminin or Matrigel can enhance some differentiation features, such as the cAMP response to FSH[37]. In the case of mammary epithelial cells, concomitant with the organizational changes induced by the ECM, gene expression and synthesis/secretion of milk proteins are also modulated (for reviews see refs 81, 138). It should however be noted that the culture of mouse mammary cells on a floating type I collagen gel allows the cells to deposit their own basement, leading subsequently to

differentiation events[157]. Thus according to these authors, the prime effect of exogenous ECM could be to regulate the production and organization of ECM by the cells themselves.

The most likely explanation for the apparent discrepancy between organs is that de novo biosynthesis by mesenchymal cells is required to form the BM in cooperation with the epithelial cells in the intestine. This idea is perfectly in accordance with in situ observations, showing a close morphological association between mesenchymal or myofibroblastic cells and epithelial cells in the intestine when intensive proliferation and differentiation occur[86,88,97]. Furthermore, coculture experiments eliminating direct contact by placing a Millipore filter in between epithelial and fibroblastic cells did not lead to enterocytic differentiation. The necessity of dynamic and reciprocal heterocellular cooperation in the intestine is also stengthened by the data obtained by Aufderheide and Ekblom[4]. Indeed, in a coculture system, tenascin expression in intestinal mesenchyme was triggered specifically by the epithelial cells.

Other arguments are brought by the effects of glucocorticoids on ECM molecules in epithelial-fibroblastic coculture experiments. Glucocorticoids are responsible for structural and enzymatic changes in the intestine mainly during development; indeed these hormones administered in vivo or added in organ cultures are able to induce precocious maturation of suckling rat intestinal epithelium (for review see refs 52, 64). In the coculture system, glucocorticoids were able to 1) modify the nature and distribution of GAGs synthesized by the fibroblastic cells, 2) induce qualitative changes in laminin molecules synthesized by mesenchyme-derived cells, 3) lead to an accelerated organization of laminin-1 at the epithelial/mesenchymal interface. These modifications accompanied the accelerated maturation of the intestinal embryonic endodermal cells[15,121]. It is important to stress that injections of glucocorticoids to 12-day-old rats led to an increase in type IV (pro)collagen, fibronectin and laminin and their respective mRNA levels; concomitantly a decrease in interstitial collagens, type I and III collagen was noted[152]. Thus, altogether these data suggest that most of the modifications brought by glucocorticoids are subsequent to changes of the ECM from stroma, strengthening the idea that fibroblastic cells are the cellular targets of the hormones. Glucocorticoids have been suggested to influence the stromal layer in other organs as well (for references see ref. 67). Recently, Ekblom et al.[42] showed that glucocorticoids could regulate hematopoiesis by modulating production of tenascin by stromal cells.

The model of heterologous coculture allowed us to provide more direct evidence of the necessity of a well-organized BM for differentiation. Indeed, the addition of polyclonal antibodies to laminin-1 to intestinal endodermal/fibroblastic cocultures led to the inhibition of expression of lactase (an apical differentiation marker). One can postulate that blockage of any other BM molecules would affect differentiation. However, several arguments from the literature point to the major role of laminin, and in particular of the constituent $\alpha 1$ chain for BM assembly, or even for morphogenesis and differentiation. Firstly, in the kidney, the induction of epithelial polarization was inhibited by antibodies towards the COOH-terminal end of laminin $\alpha 1$ chain[72]. Closely similar data were obtained in the lung, although in this case the anti-laminin antibodies that inhibited branching morphogenesis were directed to the cross region of laminin and the globular domains of the $\beta 1/\gamma 1$ chains[118]. Secondly, laminin polymeric networks, formed through relatively low affinity interactions, have been found to make a major contribution to BM architecture[157]. Thirdly, during early embryogenesis some basement membranes possess laminin, but lack type IV collagen[76]. These observations as well as the peculiar location of the $\alpha 1$ chain in the intestine, led us to analyze the role of this laminin constituent using a gene transfer strategy. The expression of antisense RNA in cells is currently widely used for reducing the expression of a targeted polypeptide[28]. This method is particularly useful for the study of vital genes whose elimination by homologous recombination is often lethal. As a model system, we used the Caco2 cells (colonic cancer cells lines) expressing non negligable levels of laminin $\alpha 1$ chain; these cells exhibit phenotypic markers of human fetal small intestinal cells[100]. Immunoblot and immunocytochemistry analysis revealed that the three constituent chains of laminin – $\alpha 1$, $\beta 1$ and $\gamma 1$ – were expressed in Caco2 cells, whereas another colonic cancer cell line, HT29, did not express detectable levels of $\alpha 1$ chain[33]. When cultured on top of fibroblastic cells, Caco2 cells grew as monolayers. At the Caco2 cells/fibroblast interface, a continuous BM was noted as assessed by electron microscopy and immunodetection of collagen IV and laminin. In contrast, the HT29 grew as clusters on fibroblastic cells and no polar deposition of any BM components occurred at regions where HT29 cells and fibroblasts were confronted[14]. The synthesis of laminin was the highest in Caco2 cells as compared to HT29 cells. To study the effect of reduced laminin $\alpha 1$ chain expression on cell behavior, Caco2 cells were transfected with an antisense laminin $\alpha 1$-E3 fragment cDNA construct; clones displaying decreased laminin $\alpha 1$ levels were isolated. As a consequence, these clones accumulated $\beta 1/\gamma 1$ chains intracellularly. In coculture experiments, these deficient cells did not deposit a laminin-containing matrix in contact with fibroblasts; in parallel no collagen IV was found[32]. These preliminary data allowed us to conclude that the $\alpha 1$ chain of laminin contains information required for the formation of a stable laminin-containing basement membrane and for the complete assembly of the matrix network.

Concluding remarks

Morphogenesis and differentiation, which are the processes by which cells 1) shape the detailed architectural features of tissues, and 2) acquire their tissue-specific functions are characterized by a complex cascade of cellular and biochemical events under precise regulatory controls. Based on the observations and data reported herein we propose that the continuous cell-cell and cell-matrix interactions, during intestinal development and adult cell renewal, are the main driving forces involved in these processes. These data stress the fact that not only epithelial cells but also mesenchymal-like cells act as crucial regulators, together with the ECM which is a highly structured entity continuous with the cell surface and cell interior.

Although several elegant contributions have allowed significant progress in the understanding of gastro-intestinal cell biology[46], few studies have focused on the molecular mechanisms governing the integrated epithelium-mesenchyme unit in the onset and maintenance of gut morphogenesis and differentiation. The precise knowledge of the tissular, cellular and extracellular matrix variations in various tissue systems has led to the vast field of research concerning molecular events involved in these changes. The most important questions in the near future will be to know how 1) the expression of ECM molecules and of their receptors as well as of the degradation enzymes in a given tissue is controlled, 2) tissue- or cell-specific gene expression is regulated by the extracellular microenvironment. As most of these aspects have been and will be approached in cell culture systems, a major challenge will also be to define the actual role in vivo of individual ECM molecules in tissue development and functional maintenance.

Linked to the first question, it is worth noting that the tissue-specific expression, developmental control and coordinate synthesis of the different chains of a given ECM molecule must imply complex interactions of multiple regulatory proteins. In several recent studies, various effectors have been shown to act on ECM production. Among them are glucocorticoids known (see previous section) to influence intestinal maturation through their effect on ECM production by the mesenchymal cells. In a closely similar differentiation inductive system (hematopoietic differentiation), Ekblom et al.[42] have speculated that the hormone-receptor complex acts directly on *cis*-regulatory elements of the targeted gene (tenascin gene in this particular case). Another example of ECM regulation is given by the discovery of retinoic acid (a known morphogen-differentiation inducer)-responsive elements in the laminin $\beta 1$ and type IV collagen promoters, that may modulate gene transcription[19,149]. Moreover, the discovery of a novel specific sequence motif that appears to be unique to the regulatory regions of many genes encoding BM proteins, suggests that it represents an important and potentially unique control element for the coordinate expression and regulation of ECM[illegible].

ECM (like cell adhesion protein) gene promoters also contain control elements which are targets for homeobox gene products (described for the cytotactin tenascin gene[55]). The huge number of studies performed during the last decade on these homeotic genes has clearly shown that they are involved in the establishment of the antero-posterior axis in multicellular organisms, and in the patterning of particular tissues. In the intestinal tissue, the following observations are of interest. Firstly, there is a differential expression gradient of several homeotic genes from the anterior small intestine to the distal colon[43,57]. Secondly, the overexpression of the homeobox-containing gene *Hoxa*-4 in transgenic mice led to the abnormal development of the smooth muscle coat and innervation of the terminal gut (megacolon); these alterations were paralleled by an abnormal deposition of BM molecules[139]. Considering those data, it is tempting to speculate that this gene acts on downstream morphogenetic effector genes which could encode ECM molecules.

Cytokines are also good candidates for regulating the extracellular matrix steady state. Among them, TGFβ has been shown to act on the transcription of variety of matrix components and also to up-regulate integrins and inhibit degradation of extracellular matrix[31,101]. More insight into the molecular mechanisms of TGFβ function begins to emerge; for example, activation of collagen transcription occurs through a specific activation element[107]. A variety of other cytokines, acting either on epithelial or on mesenchymal tissues, also display quite marked effects on compositional changes of the entire ECM. The properties of these cytokines have been particularly well analyzed in inflammatory diseases (with an increasing attention towards chronic inflammatory bowel diseases), where they are involved in immunocompetent cell recruitment, fibroblast proliferation and tissue remodeling[93,117,151]. An additional level of cell regulation is brought by the ability of cytokines to bind directly to ECM molecules[1,94]; in this way, ECM molecules function as reservoirs for various growth factors that can be either immediately available to cells when need arises, or undergo conformational changes allowing them to get access to their receptors. Finally, a link between growth factors and homeobox gene expression could mediate signaling implicated in epithelial-mesenchymal interactions which do not necessarily imply direct contact between tissues. Two recent studies examplify such an interplay. Vainio et al.[146] showed in tooth rudiment that BMP-4 (bone morphogenetic protein 4), a member of the TGFβ superfamily, was able to replace the epithelium in inducing morphological and molecular changes in the dental mes-

enchyme, leading to tooth induction. Furthermore, BMP-4 induced the expression in the presumptive dental mesenchyme of *Msx*-1 and *Msx*-2 homeobox genes which may be involved in the establishment of positional information during tooth organogenesis[146]. In the adult mouse uterus, the expression of *Msx*-1 in the epithelium was shown to be induced by the underlying mesenchyme. This property of the uterine mesenchyme is correlated with the mesenchymal expression of *Wnt*-5, the vertebrate homologue of the Drosophila wingless family of growth factors[99]. Thus, the combination of growth factor-like components and of homeobox-containing genes could play a role in the developmental patterning as well as in maintenance of the adult organ in a morphogenetic responsive state.

Concerning the second important question raised above which dealt with the cellular response to the extracellular microenvironment, the general hypothesis for the mechanism by which ECM regulates gene expression implies the following steps. The first one consists of binding of ECM to specific receptors leading to their clustering. Subsequently, a number of more or less well defined intracellular modifications will occur. The signal transduction, starting from changes in the cytoplasmic domain of the receptors, may involve either changes in the state of assembly of the cytoskeleton or the action of various potential second messengers, implying mostly phosphorylation events (for reviews see refs 1, 60). The final step of the intracellular transduction cascade, whatever its nature, deals with the transcriptional regulation of the responsive genes. A recent review by Lin and Bissell[81] summarizes the current knowledge about this latter aspect in mammary cells, hepatocytes and keratinocytes. They report the presence of ECM/hormone-responsive enhancers in the promoter region of tissue-specific genes, and the activation of specific transcription factors by extracellular signals. The mitogenic response of the C-terminal end of laminin $\alpha 1$ chain long arm includes, as in the response to growth factors, a rapid and transient increase of c-fos and c-jun protooncogenes expression, and DNA binding activity[74].

Almost all the emerging data on the role of individual ECM molecules and on the molecular mechanisms involved in the integrated epithelial-ECM-mesenchymal unit arise from in vitro studies; a major challenge will be now to confirm these findings in situ. An approach to this fundamental question in cell biology and physiology is to analyze the consequences of the overexpression or knock-out of a given molecule on morphogenesis and differentiation processes. Unfortunately, most of the mutations giving deficient expression of ECM molecules or receptors are lethal due to the impairment of major morphogenetic steps in early embryogenesis; thus it is impossible to draw any conclusion about their function in the intestinal tissue. Up to now, two ECM molecule-deficient models have been examined: tenascin knock-out[119] and merosin deficient *dy* mutant mouse[26,155]. They did not reveal any significant modification of the intestinal morphogenesis or crypt villus formation. Tissue-specific and temporally targeted alterations must now be performed to overcome lethality in transgenic mice or functional redundancy of some of these components.

In conclusion, the benefit of many integrated investigations performed in a wide range of disciplines will most probably lead in the near future to a better understanding of the mechanisms involved in cell-cell communication and of their implication in cell biology, physiology and pathology.

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Gene expression profiles in thyroid carcinomas

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Summary The gene expression profiles of human thyroid carcinomas were analysed by serial analysis of gene expression (SAGE) which allows quantitative and simultaneous analysis of a large number of transcripts. More than 29 000 transcripts derived from a normal thyroid tissue and four thyroid tumours were analysed. While extensive similarity was noted between the expression profiles of the normal thyroid tissue and three differentiated thyroid tumours, many transcripts, such as osteonectin, a-tubulin, glyceraldehyde-3-phosphate dehydrogenase, glutathione peroxidase, and thyroglobulin, were expressed at extremely different levels in differentiated and undifferentiated carcinomas. These data provide new information that might be used to identify genes useful for the diagnosis and treatment of thyroid carcinomas. © 2000 Cancer Research Campaign http://www.bjcancer.com

Keywords: gene expression; SAGE; gene therapy; anaplastic carcinoma; follicular carcinoma; molecular-based diagnosis

Recent advances in molecular technology suggest the potential for more efficient and effective molecular-based diagnoses and therapies. Many studies, such as those concerning *p53*, *RAS*, *RET*, and thyrotropin receptor, have improved our understanding of thyroid carcinogenesis (Farid, 1996). However, more intensive studies to further clarify the molecular mechanism of carcinogenesis are necessary before we select the molecular targets for these technologies.

In the thyroid, as in other organs, genes that are found to be differentially expressed between normal thyroid tissue and thyroid carcinomas can be used as targets for molecular-based diagnosis and therapy (Chiappetta et al, 1998; Takano et al, 1998, 1999). Recent developments in technologies aimed at identifying differentially expressed genes, such as differential hybridization and differential display, have identified some genes the expression of which is restricted to thyroid carcinomas (Gonsky et al, 1997; Takano et al, 1997; de Nigris et al, 1998). However, the data made available by these methods are still insufficient for a comprehensive evaluation of all genes involved in carcinogenesis.

By relying on 14–15 base cDNA sequences for gene identification, serial analysis of gene expression (SAGE) can generate a quantitative transcript profile easily, a task currently not possible using alternative transcript imaging technologies (Velculescu et al, 1995), and is less laborious than the body mapping method which can generate similar profiles (Matsubara and Okubo, 1993). Since its introduction in 1995, SAGE has been used to analyse cDNA libraries derived from several carcinomas and its reliability has been established (Zhang et al, 1997; Hibi et al, 1998). We describe here the use of SAGE to provide gene expression profiles in normal thyroid and thyroid tumours, a technique that may lead to an enhanced understanding of thyroid cell function and carcinogenesis.

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MATERIALS AND METHODS

Materials

Tissue samples for SAGE were obtained surgically from a normal thyroid tissue adjacent to a follicular adenoma in a 43-year-old female, a follicular adenoma in a 43-year-old female, a papillary carcinoma in a 32-year-old female, a widely invasive follicular carcinoma in a 35-year-old female, and an anaplastic carcinoma in a 77-year-old female. Tissue samples from three normal thyroids, follicular adenomas, papillary carcinomas, follicular carcinomas and anaplastic carcinomas were also collected for reverse transcription-polymerase chain reaction (RT-PCR) analysis. Thyroid tumours were classified histopathologically according to the WHO histological classification of thyroid tumours (Hedinger et al, 1989). Total cellular RNA was extracted according to the method of Chomczynski and Sacchi (Chomczynski and Sacchi, 1987) and poly A RNA was purified with oligotex-dT30 (Takara, Shiga, Japan) according to the manufacturer's protocol.

SAGE protocol

The SAGE method was performed as described previously with some modifications. 3 μg of poly A RNA was converted to double-stranded cDNA with a BRL synthesis kit (Gibco BRL, Tokyo, Japan) according to the manufacturer's protocol except for the inclusion of primer biotin-5′-T_{18}-3′. The cDNA was cleaved with Nla III (anchoring enzyme) (Daiichi-Kagaku, Tokyo, Japan). After capture of the 3′ cDNA fragments on streptavidin-coated magnetic

beads (Dynal, Tokyo, Japan), the bound cDNA was divided into two pools, and one of the following linkers containing a recognition site for Bsm FI (Daiichi-Kagaku) was ligated to each pool:

linker 1, 5′-TTTGGATTTGCTGGTGCAGTACAACTAG-GCTTAATAGGGACATG-3′, 5′-TCCCTATTAAGCC-TAGTTGTAXTGCACCAGCAAATCC (amino modification C7)-3′;
linker 2, 5′-TTTCTGCTCGAATTCAAGCTTCTAACGATG-TACGGGGACATG-3′, 5′-TCCCCGTACATCGTTA-GAAGCTTGAATTCGAGCAG (amino modification C7)-3′.

Since Bsm FI (tagging enzyme) cleaves 14 bp away from its recognition site, and the Nla III site overlaps the Bsm FI site by 1 bp, a 15 bp SAGE tag was released with Bsm FI. SAGE tag overhangs were filled in with Klenow (Takara), and tags from the two pools were combined and ligated to each other. The ligation product was amplified by 15 cycles of PCR using 5′-GGATTTGCTGGTGCAGTACA-3′ and 5′-CTGCTCGAAT-TCAAGCTTCT-3′ as primers. All the linkers and primers were obtained from Gibco BRL. The PCR products were analysed by polyacrylamide gel electrophoresis (PAGE), and the PCR product containing two tags ligated tail to tail (ditag) was excised. The PCR product was re-amplified by 20 cycles of PCR using the same primers, purified by PAGE, then cleaved with Nla III. The band containing the ditags was excised and self-ligated, then cleaved with Sph I (Takara). The concatenated products were separated by gel filtration using a Sephadex 400R (Amersham Pharmacia, Tokyo, Japan), then cloned into the Sph I site of pGEM-5Zf (+) (Promega, Tokyo, Japan). These procedures produced about 500 white colonies per reaction. Colonies were screened for inserts by PCR using primers which sequences located outside the cloning site. Colonies containing inserts of about 400 bp in length were selected for the further analysis. Plasmids from selected clones were purified by an automatic plasmid isolation system PI-100Σ (Kurabo, Osaka, Japan) then sequenced with Taq FS Dye Primer kits (PE Biosystems, Tokyo, Japan) and analysed using a 373 ABI automated sequencer (PE Biosystems), following the manufacturer's protocol. Sequence files were analysed by the SAGE software and the tag sequences were analysed by the BLAST program of the DNA Data Bank of Japan (Mishima, Sizuoka, Japan). The occurrence rates of tag sequences were calculated by dividing the number of occurrences of a particular tag sequence by the total tag count.

Semi-quantitative RT-PCR analysis

Semi-quantitative RT-PCR analyses of 4 representative mRNA sequences were performed as previously described (Takano et al, 1997). The sequence of the 5′ primers are 5′-GGATTTGCTGGT-GCAGTACA-3′ (base 1511–1530) (Swaroop et al, 1988) for

Table 1 SAGE analysis of a normal thyroid and a follicular adenoma

Normal thyroid
Total no. of tags = 5411, no. of unique tags = 623

Count	Sequence	Definition
64	CCACTGCACT	EST A1081056
63	CGGTGAAAAA	thyroglobulin
55	ACTTTTTCAA	mitochondrial cytochrome oxidase subunit 1
50	GTGAAACCCC(G)	1. Alu transcript 2. obese protein 3. platelet-activating factor acetylhydrolase 2
49	CCTGTAATCCC	5′-nucleotidase
48	GTGAAACCCC(A)	1. granulocyte-macrophage colony-stimulating factor receptor alpha-subunit soluble isoform 2 2. myelin/oligodendrocyte glycoprotein-25.1kD 3. fibroblast growth factor receptor
33	GTGAAACCCT	putative serine-thereonine protein kinase
30	TGTGTTGAGA	elongation factor 1-alpha
29	CACCTAATTG	mitochondrial ATP synthase 6
28	AACCCGGGAG	1. transmembrane receptor protein 2. primary Alu transcript
28	CCCATCGTCC	mitochondrial cytochrome oxidase subunit 2
26	AGGGAGGGGC	glutathione peroxidase
25	TCAAGCCATC	EST AI563994
25	TTCATACACC	mitochondrial NADH dehydrogenase 4
23	CTCCACCCGA	secretory protein
23	TACATAATTA	trophoblast STAT
20	AGCCCTACAA	mitochondrial NADH dehydrogenase 3
20	GCGAAACCCC	EST N71314
19	AACCTGGGAG	DNA fragmentation factor-45
19	AGCTCTCCCT	putative ribosomal protein L23
18	CTAAGACTTC	EST C04521
18	TTGGCTTGCT	EST AA515148
15	ACCCTTGGCC	mitochondrial NADH dehydrogenase 1
15	AGGTCAGGAG	human carcinoma cell-derived Alu RNA transcript, clone CD139
15	CAAGCATCCC	EST A1557493
15	CGCCGCCGGC	ribosomal protein L35

Follicular adenoma
Total no. of tags = 5030, no. of unique tags = 569

Count	Sequence	Definition
144	CGGTGAAAAA	thyroglobulin
56	CCTGTAATCC	5′-nucleotidase
54	ACTTTTTCAA	mitochondrial cytochrome oxidase subunit 1
54	CCACTGCACT	EST AI081056
47	CGGTGAAGCA	no match
38	GTGAAACCCT	putative serine-threonine protein kinase
34	TGTGTTGAGA	elongation factor 1-alpha
33	GTGAAACCCC(G)	1. Alu transcript 2. obese protein 3. platelet-activating factor acetylhydrolase 2
29	GTGAAACCCC(A)	1. granulocyte-macrophage colony-stimulating factor receptor alpha-subunit soluble isoform 2 2. myelin/oligodendrocyte glycoprotein-25.1kD 3. fibroblast growth factor receptor
23	TAGGTTGTCT	translationally controlled tumor protein
20	CACCTAATTG	mitochondrial ATP synthase 6
19	AGCTCTCCCT	putative ribosomal protein L23
19	GGCAAGCCCC	Csa-19
19	TTCATACACC	mitochondrial NADH dehydrogenase 4
19	TTGGTCCTCT	ribosomal protein L41
18	CCCATCGTCC	mitochondrial cytochrome oxidase subunit 2
17	AACCCGGGAG	1. transmebrane receptor protein 2. primary Alu transcript
17	AGCCCTACAA	mitochondrial NADH dehydrogenase 3
16	CCTCAGGATA	mitochondrial NADH dehydrogenase 6
16	GCCGAGGAAG	ribosomal protein S12
15	CACAAACGGT	1. metallopanstimulin 2. ribosomal protein S27
14	CCTGTAGTCC	EST R10346
14	TTGGCCAGGC	1. aggrecanase-1 2. interferon-inducible RNA-dependent protein kinase 3. glucose-6-phosphatase
13	CCAGAACAGA	1. ribosomal protein L30 2. thymidylate kinase
14	GACGACACGA	ribosomal protein S28

Table 2 SAGE analysis of papillary, follicular and anaplastic carcinomas

Papillary carcinoma
Total no. of tags = 6435, no. of unique tags = 662

Count	Sequence	Definition
159	CCCATCGTCC	mitochondrial cytochrome oxidase subunit 2
146	CACCTAATTG	mitochondrial ATP synthase 6
122	ACCCTTGGCC	mitochondrial NADH dehydrogenase 1
93	TGATTTCACT	mitochondrial cytochrome c oxidase subunit 3
84	TTGGGGTTTC	ferritin H chain
79	GTGAAACCCC(G)	1. Alu transcript 2. obese protein 3. platelet-activating factor acetylhydrolase 2
77	ACTAACACCC	mitochondrial NADH dehydrogenase 2
65	TTCATACACC	mitochondrial NADH dehydrogenase 4
64	TTGGTCCTCT	ribosomal protein L41
56	ACTTTTTCAA	mitochondrial cytochrome oxidase subunit 1
56	CAAGCATCCC	EST AI557493
56	CACTACTCAC	mitochondrial cytochrome b
51	AGCCCTACAA	mitochondrial NADH dehydrogenase 3
46	TGTGTTGAGA	elongation factor 1-alpha
44	CCACTGCACT	EST AI081056
44	CCTGTAATCC	5'-nucleotidase
37	CTAAGACTTC	EST C04521
35	CGGTGAAAAA	thyroglobulin
33	TCGAAGCCCC	EST AA533220
31	GCCGAGGAAG	ribosomal protein S12
30	TGGGTGAGCC	cathepsin B
28	AAGACAGTGG	ribosomal protein L37a
27	AGCACCTCCA	elongation factor 2
26	GGACCACTGA	ribosomal protein L3
23	GCAGCCATCC	ribosomal protein L28

Follicular carcinoma
Total no. of tags = 5275, no. of unique tags = 630

Count	Sequence	Definition
188	CGGTGAAAAA	thyroglobulin
55	CCTGTAATCC	5'-nucleotidase
51	CCCATCGTCC	mitochondrial cytochrome oxidase subunit 2
45	AGCCCTACAA	mitochondrial NADH dehydrogenase 3
44	CACCTAATTG	mitochondrial ATP synthase 6
38	CCACTGCACT	EST AI081056
34	GTGAAACCCC(G)	1. Alu transcript 2. obese protein 3. platelet-activating factor acetylhydrolase 2
30	CGGTGAAGCA	no match
29	AGGGAGGGGC	glutathione peroxidase
29	TTGGTCCTCT	ribosomal protein L41
28	AGGGCTTCCA	Wilm's tumor-related protein
26	ACCCTTGGCC	mitochondrial NADH dehydrogenase 1
26	TGTGACGCCG	no match
23	ACTAACACCC	mitochondrial NADH dehydrogenase 2
23	TGGGTGAGCC	cathepsin B
21	CACAAACGGT	1. metallopanstimulin 2. ribosomal protein S27
20	AAGACAGTGG	ribosomal protein L37a
20	CGCCGCCGGC	ribosomal protein L35
20	GTGAAACCCT	putative serine-threonine protein kinase
19	TGTGTTGAGA	elongation factor 1-alpha
19	TTCATACACC	mitochondrial NADH dehydrogenase 4
18	GTGAAACCCC(A)	1. granulocyte-macrophage colony-stimulating factor receptor alpha-subunit soluble isoform 2 2. myelin/oligodendrocyte glycoprotein-25.1kD 3. fibroblast growth factor receptor transmembrane form
18	AACCCGGGAG	1. transmebrane receptor protein 2. primary Alu transcript
18	CCATTGCACT	EST T07339
18	GCAGCCATCC	ribosomal protein L28

Anaplastic carcinoma
Total no. of tags = 7124, no. of unique tags = 849

Count	Sequence	Definition
87	ACTTTTTCAA	mitochondrial cytochrome oxidase subunit 1
64	CCCATCGTCC	mitochondrial cytochrome oxidase subunit 2
63	GTTGTGGTTA	beta 2-microglobulin
60	ATGTGAAGAG	*SPARC*/osteonectin
59	TTCATACACC	mitochondrial NADH dehydrogenase 4
55	TGGAAATGAC	alpha-1 collagen (polymorphic transcript)
51	GTTCACATTA	HLA-DR antigens associated invariant chain
41	AGCCCTACAA	mitochondrial NADH dehydrogenase 3
41	CACCTCCTAT	no match
40	GGATTTGGCC	acidic ribosomal phosphoprotein P2
40	GTGTGTTTGT	transforming growth factor beta induced gene product
37	ACCAAAAACC	alpha-1 collagen type 1
37	CCAGAACAGA	1. ribosomal protein L30 2. thymidylate kinase
34	CCTAGCTGGA	T-cell cyclophilin
32	GAGGGAGTTT	ribosomal protein L27a
29	TACCATCAAT	glyceraldehyde-3-phosphate dehydrogenase
28	CCACTGCACT	EST AI081056
28	TTGGGGTTTC	ferritin H chain
27	AGGGCTTCCA	Wilm's tumour related protein
27	CACCTAATTG	mitochondrial ATP synthase 6
27	CCCTGGGTTC	ferritin light subunit
27	TAGGTTGTCT	translationally controlled tumour protein
27	TTGGTCCTCT	ribosomal protein L
26	AAGGTGGAGG	ribosomal protein L18a
26	AGAAAAAAAA	no match

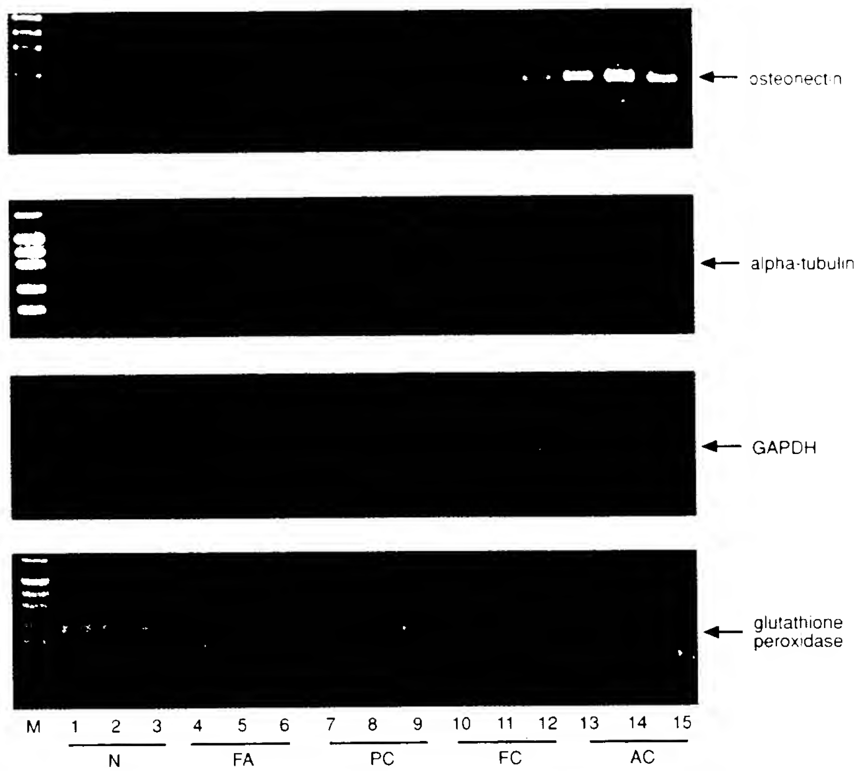

Figure 1 Semi-quantitative RT-PCR analysis of osteonectin, α-tubulin, GAPDH, and glutathione peroxidase mRNAs. Tissue samples from three normal thyroids (N), follicular adenomas (FA), papillary carcinomas (PC), follicular carcinomas (FC) and anaplastic carcinomas (AC) were subjected to RT-PCR analysis. PCR products were run on a 1.5% agarose gel, then the gel was stained with SYBR Green 1 (Takara). Arrows indicate the expected positions of the PCR products. M: PHY maker (Takara)

osteonectin, 5′-GGATTTGCTGGTGCAGTACA-3′ (base 1021–1040) for α-tubulin (Cowan et al, 1983), 5′-CCAAGGTCATCCATGACAAC (base 557–576) for glyceraldehydes-3-phosphate dehydrogenase (GAPDH) (Arcari et al, 1984), and 5′-ACGTGTCCTACCTATGTGTC-3′ (base 981–1000) for glutathione peroxidase (Takahashi et al, 1990). A poly A-anchor primer DDR (5′-ATGCGAATTCGTTTTTTTTTTTTTTTTTTT-3′) was used for the 3′ primer. RT was performed using 1 μg of total RNA in an RT mixture containing 40 mM Tris-HCl (pH 8.3), 75 mM KCl, 10 mM DTT, 3 mM $MgCl_2$, 0.5 mM dNTPs, 200 U Moloney murine leukaemia virus reverse transcriptase (Gibco BRL), 2 U/μl RNase inhibitor (Takara), and 2.5 μM oligodeoxythymidylic acid (Gibco BRL) in a total volume of 20 μl at 37°C for 60 min. For PCR, each reaction mixture consist of 1 μl of cDNA, 0.5 μM each primer, 2 μl of 10 × Ex Taq buffer (Takara), 1.6 μl of 2 mM dNTP mix (PE Biosystems) 0.5 U of Ex Taq polymerase (Takara), and nuclease-free water to a final volume of 20 μl. The reaction mixture was subjected to 25 cycles of denaturation (94°C; 1 min), annealing (55°C; 1 min), andextension (72°C; 1 min). After PCR amplification, 5 μl of reaction mixture was run on 1.5% agarose gel. The gel was stained with SYBR Green I (Takara), then analysed with a Fluor Imager (Molecular Dynamics, Sunnyvale, CA).

RESULTS

SAGE libraries were constructed from mRNAs isolated from a normal thyroid tissue sample and four thyroid tumours. In total, 29 275 tags were sequenced, representing about 600 unique tags in each tissue (Tables 1 and 2). The majority of the highly expressed sequences in each tissue code mitochondorial and ribosomal proteins. The tag sequence of thyroglobulin mRNA was highly expressed in the normal thyroid and the 3 differentiated thyroid tumours but not in the anaplastic carcinoma. In the 2 differentiated carcinomas, high expression levels of the tag sequence of cathepsin B were observed. In the anaplastic carcinoma, most of the highly occurring tag sequences were derived from housekeeping genes in addition to mitochondorial and ribosomal sequences. Some sequences that were only seldom observed in the differentiated carcinomas, such as those of osteonectin and collagen genes, were also highly expressed.

To generate a profile of the relative gene expression patterns in each tumour, the occurrences of each tag identified in the tumour library were compared with those observed in the libraries of the other tumours or of the normal thyroid. Representative sequences are listed in Tables 3 and 4. The tag sequences that code mitochondorial and ribosomal proteins were excluded from the lists. A small number of tag sequences showed extreme differences in the expression levels among the normal thyroid and differentiated tumours. In contrast, among the 97 tag sequences which occurred 10 times or more, 29 (29.8%) and 27 (27.8%) sequences occurred at rates 10-fold or more than those in papillary and follicular carcinomas, respectively, which indicates that the expression profile of the anaplastic carcinoma is much different from those of the differentiated carcinomas.

Expression levels of some genes whose tag sequences were differentially expressed in the anaplastic carcinoma were examined by semi-quantitative RT-PCR. Semi-quantitative RT-PCR

Table 3 List of differentially expressed genes in the normal thyroid (N), follicular adenoma (F), papillary carcinoma (PC), and follicular carcinoma (FC)

Count		
N	F	Sequence
25	0	TCAAGCCATC
EST AI563994		
18	0	TTGGCTTGCT
EST AA515148		
14	1	GAAATAAAGC
germline immunoglobulin gamma 1 chain constant region		
14	0	CCCAACGCGC
alpha globin		
13	1	AAGGGAGCAC
Ig germline lambda-chain		
13	1	GGATATGTGG
transcription factor ETR103		
n = 6		

Count		
F	PC	Sequence
1	20	GCAAGCCAAC
EST AA133564		
1	18	ACACAGCAAG
EST AA654674		
1	17	GCGACCGTCA
aldolase A		
0	13	ACCTTGTGCC
L-iditol-2 dehydrogenase		
0	13	GCCATCCCCT
mRNA from HIV-associated non-Hodgkin's lymphoma (clone h12-129)		
0	11	ATGGCTGGTA
LLRep3		
0	10	CTGACCTGTG
MHC HLA-B7 class I cell surface glycoprotein heavy chain		
n = 12		

Count		
F	FC	Sequence
0	26	TGTGACGCCG
no match		
23	3	TAGGTTGTCT
translationally controlled tumour protein		
0	14	GGAGGTGGG
1. granulin 2. epithelin 1 and 2		
0	13	GGGGAAATC
thymosin beta 10		
0	10	ACCAAAAACC
alpha-1 collagen type 1		
n = 5		

n: the number of sequences occurred at rates ten-fold or more than those in the compared tissue.

Table 4 List of differentially expressed genes in the papillary (PC), follicular (FC), and anaplastic (AC) carcinomas

Count PC	Count FC	Sequence	Gene
0	26	TGTGACGCCG	no match
11	0	AAAACATTCT	EST AA095120
10	0	CTGACCTGTG	MH C HLA-B7 class I cell surface glycoprotein heavy chain
n = 7			

Count PC	Count AC	Sequence	Gene
4	60	ATGTGAAGAG	*SPARC*/osteonectin
8	51	GTTCACATTA	HLA-DR antigens associated invariant chain
0	55	TGGAAATGAC	alpha-1 collagen (polymorphic transcript)
0	40	GTGTGTTTGT	transforming growth factor-beta induced gene product
0	37	ACCAAAAACC	alpha-1 collagen type 1
33	4	TCGAAGCCCC	EST AA533220
35	0	CGGTGAAAAA	thyroglobulin
2	29	TACCATCAAT	glyceraldehyde-3-phosphate dehydrogenase
1	26	TTGACACTTT	no match
0	26	AGAAAAAAAA	no match
2	23	GGGCATCTCT	HLA-DR alpha-chain
2	21	TGTACCTGTA	alpha-tubulin
1	21	CTTGTAATCC	EST H87461
n = 45			

Count FC	Count AC	Sequence	Gene
188	0	CGGTGAAAAA	thyroglobulin
5	63	TTGTGGTTAA	beta 2-microglobulin
3	60	ATGTGAAGAG	SPARC/osteonectin
4	51	GTTCACATTA	HLA-DR antigens associated invariant chain
0	41	CACCTCCTAT	no match
0	40	GTGTGTTTGT	transforming growth factor-beta induced gene product
1	29	TACCATCAAT	glyceraldehyde-3-phosphate dehydrogenase
0	26	AGAAAAAAAA	no match
0	26	TTGACACTTT	no match
30	0	CGGTGAAGCA	no match
29	0	AGGGAGGGGC	glutathione peroxidase
1	21	TGTACCTGTA	alpha-tubulin
1	21	TTGCTGACTT	collagen VI alpha-1
n = 34			

n: the number of sequences occurred at rates ten-fold or more than those in the compared tissue.

confirmed increased expression of osteonectin, α-tubulin, and GAPDH, and decreased expression of glutathione peroxidase mRNA in 3 anaplastic carcinomas (Figure 1).

DISCUSSION

In this study, we used SAGE to analyse cDNAs from tissues of a normal thyroid and 4 thyroid tumours and created expression profiles for each tissue. In our results, some tag sequences corresponded to more than one gene. It was not possible, by means of only SAGE-data analysis, to determine whether all of the corresponding genes were expressed in the tissue. In the case of these sequences, further analyses, such as Northern blot or quantitative RT-PCR analyses, may be needed. Some tag sequences with no homology to known genes appear on the list. These sequences might be derived from some unknown genes, although the possibility of interference by the individual variations in the 3′ untranslated region of mRNAs should be also considered.

Pauws et al recently described the application of SAGE to create an expression profile of the normal thyroid (Pauws et al, 2000). Their data are quite similar to ours in that the majority of the highly expressed sequences coded mitochondorial or ribosomal proteins and the thyroglobulin gene was highly expressed. However, while they detected 24 tags of thyroid peroxidase, we detected none in the normal thyroid tissue and only 2 in the follicular adenoma. The effects of some endemic factors, such as iodine uptake, may explain this discrepancy. Further, because we performed SAGE analysis on a smaller scale than they did, only about 600 unique genes were identified. Thus, the analyses were limited to abundantly expressed sequences, and this is another reason why most of the thyroid-specific genes with moderate or low expression levels could not be detected.

In our study, the tag sequences of some genes, such as thyroglobulin, cathepsin B, and thymosin beta 10, were expressed in the benign and malignant tumours in a manner similar to that in previous reports (Brabant et al, 1991; Shuja and Murnane, 1996; Califano et al, 1998), suggesting the reliability of these SAGE data. For example, the tag sequence of cathepsin B occurred at a much higher rate in the papillary and follicular carcinomas than in the normal thyroid or the follicular adenoma.

In the anaplastic carcinomas, most of the highly occurring tag sequences code mitochondorial proteins, ribosomal proteins, or housekeeping genes, such as GAPDH. Interestingly, the products of some of these genes are already being used as serum tumour markers such as beta 2-microglobulin and ferritin. Thus, some of the genes identified here and shown to have high occurrence rates in thyroid carcinomas might be used as serum tumour markers of thyroid malignancies.

Osteonectin is a bone matrix protein synthesized by cells of the osteoblastic lineage, with a possible association having been suggested between this protein and microcalcifications in some malignant tissues (Bellahcene and Castronovo, 1995). The corresponding tag sequence of osteonectin mRNA showed a high occurrence rate in anaplastic carcinoma, and over-expression of this gene was confirmed by semi-quantitative RT-PCR. Osteonectin expression may become a new marker of anaplastic carcinomas, and the relationship between the expression of osteonectin and these cancers' aggressive biological characteristics may provide an interesting focus of study.

One of the most difficult distinctions in thyroid pathology is the differentiation between benign follicular adenomas and follicular carcinomas (Rosai and Carcangiu, 1987). Preoperative differentiation of follicular adenomas and carcinomas by cytopathological examination is quite difficult; accordingly, there has been a concentrated effort to establish a definite molecular marker of follicular carcinoma. Although only several differentially expressed genes were identified in the present study, some of the genes with known and unknown properties as listed in Table 3 may be candidate markers of follicular carcinomas.

In conclusion, in the present report, we analysed the gene expression profiles in the normal thyroid and 4 representative thyroid neoplasms. The results of this study may provide clues toward not only the establishment of a molecular-based diagnosis and therapy, but also an improved understanding of thyroid function and tumorigenesis.

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Developmental Biology

Loss of retinoic acid receptor γ function in F9 cells by gene disruption results in aberrant *Hoxa-1* expression and differentiation upon retinoic acid treatment

(embryonal carcinoma cells/gene targeting/homeobox gene expression)

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Contributed by Pierre Chambon, July 26, 1993

ABSTRACT Retinoic acid (RA) signal transduction is believed to be mediated through several high-affinity nuclear receptors [RA receptors (RARs) and retinoid X receptors], which are members of the steroid/thyroid/vitamin D superfamily and function as transcription factors. Why multiple RARs exist and what gene targets are regulated by each of the three receptors remain compelling questions in developmental biology. Through targeted disruption of both RARγ alleles, we have identified several differentiation-specific genes that are regulated either directly or indirectly by RARγ in F9 embryonal carcinoma cells. These include genes encoding Hoxa-1 (Hox-1.6) and the extracellular matrix proteins laminin B1 and collagen type IV (α1), all of which are RA inducible in wild-type F9 embryonal carcinoma cells but are not significantly induced in the RAR$\gamma^{-/-}$ lines. In contrast, transcripts encoding Hoxb-1 (Hox-2.9) and cellular RA binding protein II (CRABPII) are activated by RA for a longer period of time in the RAR$\gamma^{-/-}$ lines compared to the wild-type F9 line. Not all RA-responsive genes are aberrantly expressed; Rex-1, RARβ, and SPARC transcripts are regulated in the RAR$\gamma^{-/-}$ lines as they are in F9 wild-type cells. Our results support the idea that each RAR may regulate different subsets of RA-responsive genes, which may explain, in part, the complex regulation of developmental processes by retinoids.

All-*trans*-retinoic acid (RA), one of the most potent natural retinoids, is both an important signaling molecule in embryonic development and cell differentiation and a useful drug for the treatment of several types of cancers (1–3). Two classes of molecules are known to modulate the actions of RA. The first class is composed of the cellular RA binding proteins I (CRABPI) and II (CRABPII), which are small, cytoplasmic, high-affinity RA binding molecules (4–7). The second class consists of the highly conserved nuclear receptors—the RA receptors (RARs) α, β, and γ and the retinoid X receptors (RXRs) α, β, and γ—that function as ligand-inducible transcription factors through the formation of heterodimers bound to specific RA response elements (RAREs) (refs. 1 and 8–10 and references therein). Both all-*trans*-RA and 9-*cis*-RA are effective ligands for the RARs, while the RXRs bind only 9-*cis*-RA with high affinity (refs. 9–11 and references therein). The existence of multiple RARs suggested that each may perform a unique function (8). This idea was supported by the observation that in the developing mouse embryo each RAR exhibits distinct spatiotemporal expression patterns (12, 13).

Thus, one of the important questions in the field of developmental biology concerns the roles that each of the three RARs and RXRs may play in transducing the RA signal. Since murine F9 embryonal carcinoma cells, when treated with RA, differentiate into endoderm cells resembling those of the mouse blastocyst, these cells represent an attractive model system in which to study the early events of mammalian development and retinoid signaling. Elimination of the RARγ protein via targeted disruption of both RARγ alleles in F9 cells affords the opportunity to study the function of the protein. In this report, we demonstrate that F9 stem cells carrying such a disruption fail to exhibit a normal differentiation morphology upon treatment with RA. At the molecular level, this abnormal differentiation is reflected by the altered expression of the transcripts of several differentiation-specific genes, including the *Hox* genes *Hoxa-1 (Hox-1.6)* and *Hoxb-1 (Hox-2.9)*, the CRABPII gene, and the genes encoding the extracellular matrix proteins laminin B1 and collagen type IV (α1). However, Rex-1 and SPARC mRNA levels remain correctly regulated by RA in the RAR$\gamma^{-/-}$ lines, showing that not all differentiation-specific genes are affected. This work identifies RA target genes that appear to be specifically regulated by one of the RARs.

MATERIALS AND METHODS

Cell Culture and Generation of Disrupted Lines. The RARγ disruption vector γ6.1 is described in detail elsewhere (14). Wild-type F9 cells (F9-Wt) were cultured under standard conditions (6); 1×10^7 cells per 500 μl suspended in electroporation buffer plus 20 μg of linearized γ6.1 plasmid were electroporated using a Bio-Rad gene pulser set at 200 V, 960 μF (15). Cells were plated at a density of 1×10^6 cells per 150-cm^2 tissue culture plate for 36 h followed by selection [G418 (300 μg/ml active drug) plus 250 μM ganciclovir] for 18–21 days. Individual colonies were isolated and propagated, and an aliquot was used for genomic Southern blot analysis. The second allele was targeted by growing the single-copy disruption line F9-Wt-γ-119 in high levels of G418 (2 mg/ml) for 21 days (15). Individual colonies were harvested and analyzed as described above.

Differentiation of F9 Stem Cells. F9-Wt, F9-Wt-γ-119, F9-Wt-γ-119-14, F9-Wt-γ-119-16, and F9-Wt-γ-119-17 stem cells were cultured and treated with 1 μM all-*trans*-RA with or without dibutyryl cAMP and theophylline as described (4, 6). No differences in doubling times were noted among the five cell lines in the presence or absence of RA (data not shown). For Northern blot analysis, the same stem cell lines were treated with 1 μM all-*trans*-RA, and RNA was prepared at the indicated times. Southern and Northern blot analyses were performed as described elsewhere (6).

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Abbreviations: RA, retinoic acid; RAR, RA receptor; RXR, retinoid X receptor; RARE, RA response element; wt, wild type.
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Gel Mobility-Shift Assay. The expression vectors for the mouse RAR coding sequences RARα1 (16, 17), RARβ2 (16), and RARγ1 (16, 18) in pSG5 (19) have been described. Cells were transfected with 10 μg of the murine RAR expression vector plus 10 μg of carrier plasmid by the calcium phosphate technique (20). Cells were grown in the presence of 1 μM all-*trans*-RA for 24 h. Whole-cell extracts from cultures of F9-Wt and F9-Wt-γ-119-14 cells were prepared as reported (21). Mobility-shift assays were performed as described by Garner and Revzin (22) using the *Hoxa-1*/RAREβ double-stranded oligodeoxynucleotide corresponding to the RARE of the *Hoxa-1* and RARβ2 genes (23, 24) as described by Nicholson *et al.* (25). Protein extracts (4 μg) were preincubated with 50,000 cpm of ^{32}P-labeled oligonucleotide probe followed by addition of 1 μl of ascites fluid monoclonal antibodies directed against the F region of RARα1, -β2, or -γ1 (21, 26, 27). The protein complexes were resolved on a 5% polyacrylamide gel.

RESULTS

The genomic sequence encoding the B domain of the RARγ protein was targeted with a replacement construct containing 6 kb of genomic sequence interrupted by the neomycin-resistance gene (Fig. 1*a*; ref. 14). Homologous recombination was detected by genomic Southern blotting with a genomic probe flanking the targeting construct. This probe gives rise to a 4.5-kb *Bam*HI genomic fragment specific for the targeting event, compared to the wild-type (wt) 6-kb *Bam*HI fragment (Fig. 1 *a* and *b*). Fig. 1*b* shows that one copy of the RARγ gene has been disrupted in the F9-Wt-γ-119 cell line. The second allele was disrupted by selecting F9-Wt-γ-119 cells in high levels of G418 (15), resulting in 4 RAR$\gamma^{-/-}$ lines of 36 G418-resistant lines tested. Two of these cell lines, F9-Wt-γ-119-14 and F9-Wt-γ-119-16, were chosen for further study (Fig. 1*b*).

To confirm the disruption of both alleles of the RARγ gene, RARγ RNA levels were measured by Northern blot analysis (Fig. 1*c*). The F9-Wt-γ-119-14 and F9-Wt-γ-119-16 cell lines do not contain RARγ-specific transcripts as compared to the control lines (Fig. 1*c*, lanes 3 and 4). Thus, both copies of the RARγ gene have been successfully disrupted by homologous recombination initiated by the γ6.1 targeting construct.

The ability of RAR$\gamma^{-/-}$ F9 cells to respond to RA was first determined. F9-Wt stem cells and the RAR$\gamma^{-/-}$ (F9-Wt-γ-

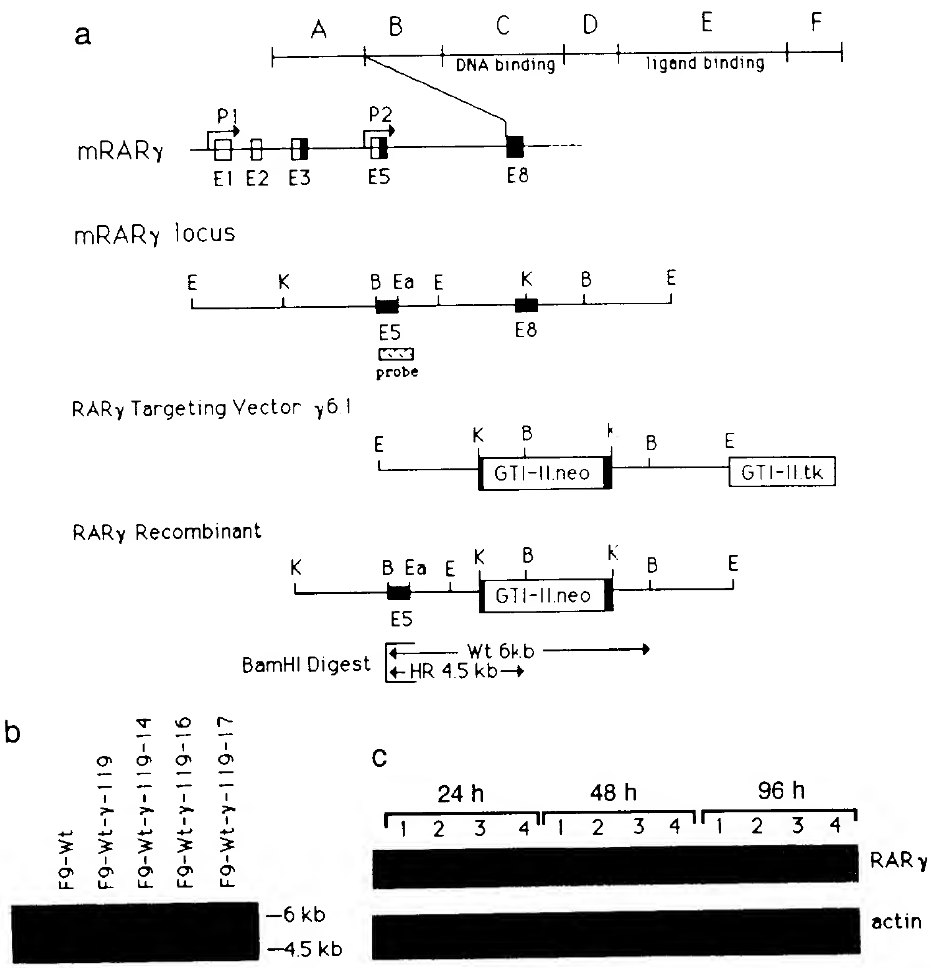

FIG. 1. Targeted disruption of the RARγ gene by homologous recombination. (*a*) The RARγ disruption vector γ6.1 is shown (14). The RARγ genomic sequence spanning the region encoding the B domain of the RARγ protein is interrupted by the neomycin-expression gene driven by the GTI-II (simian virus 40 enhancer) plus the Rous sarcoma virus TATA box (14). The herpes simplex virus thymidine kinase (tk) gene is located at the 3′ boundary of the targeting sequences. Using the probe indicated, a successful recombination event gives rise to a 4.5-kb *Bam*HI genomic fragment, in contrast to the wt 6-kb genomic fragment. This targeting vector is designed to disrupt all isoforms of the RARγ gene. For full details, see ref. 14. B, *Bam*HI; E, *Eco*RI; Ea, *Eag* I; K, *Kpn* I. (*b*) Southern blot analysis demonstrating disruption of the RARγ gene. Positions of the targeted genomic fragment and of the wt genomic fragment are indicated. Cell line F9-Wt-γ-119-17 is also included as a control since this line was derived from the high G418 selection but failed to undergo gene conversion. (*c*) Northern blot analysis showing the absence of the endogenous RARγ mRNA. Cells were treated with 1 μM RA and RNA was prepared at the indicated times. Lanes: 1, F9-Wt; 2, F9-Wt-γ-119; 3, F9-Wt-γ-119-14; 4, F9-Wt-γ-119-16. The 3- to 4-fold reduction of RARγ RNA in the control lines F9-Wt and F9-Wt-γ-119 is consistent with previously published reports (16, 28). There is no detectable RARγ RNA in the RAR$\gamma^{-/-}$ cell lines F9-Wt-γ-119-14 and F9-Wt-γ-119-16.

119-14) line possess a similar morphology in the absence of all-*trans*-RA (Fig. 2). However, in the presence of RA or RA plus dibutyryl cAMP and theophylline, the F9-Wt (Fig. 2) and RAR$\gamma^{+/-}$ (data not shown) cells change their morphology, developing pronounced cell borders and extending cellular processes associated with differentiation (Fig. 2 *a–d*). In contrast, the RAR$\gamma^{-/-}$ line exhibits fewer of the differentiation-specific morphological characteristics noted in the F9-Wt cells (Fig. 2 *a′–d′*). Interestingly, the RAR$\gamma^{-/-}$ line begins to display some of the differentiation characteristics at later times after RA treatment (data not shown). Thus, RARγ provides a critical function during the differentiation of F9 stem cells, the absence of which significantly alters the normal RA responsiveness with respect to the morphological changes associated with differentiation.

To investigate the function of RARγ during RA-induced differentiation, the two RAR$\gamma^{-/-}$ cell lines were treated with 1 μM all-*trans*-RA and the differentiation was monitored by Northern blot analysis (Fig. 3). In the lines that do not express RARγ RNA, there is minimal induction of the *Hoxa-1* gene at 24 h, and only low levels of Hoxa-1 RNA are detected at 48 h after RA treatment. Moreover, no induction of laminin B1 or collagen type IV (α1) RNAs is observed in the RAR$\gamma^{-/-}$ lines. In contrast, in the RAR$\gamma^{-/-}$ lines the

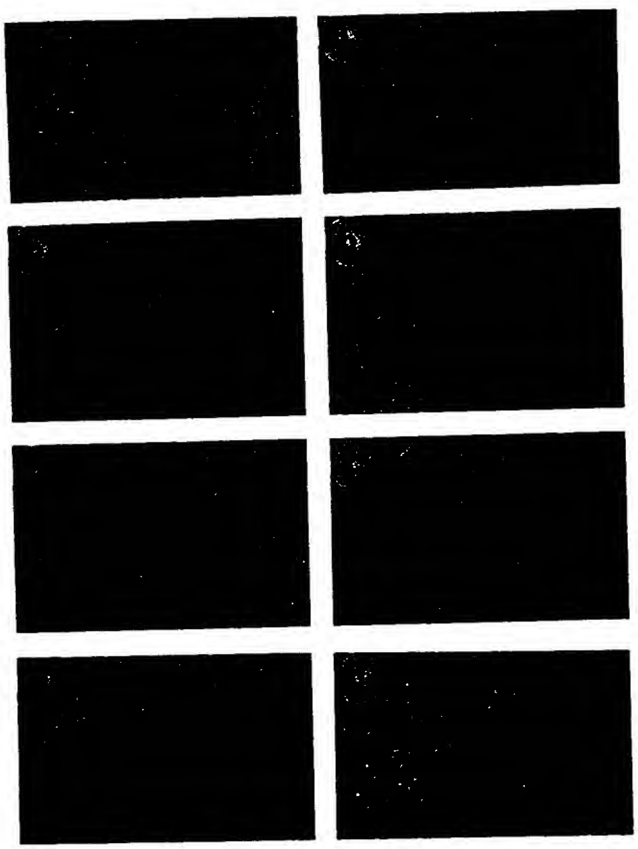

FIG. 2. RAR$\gamma^{-/-}$ cell lines fail to exhibit a complete differentiation morphology in culture. As stem cells, the F9-Wt and the RAR$\gamma^{-/-}$ cell line F9-Wt-γ-119-14 share a similar compact morphology. However, after treatment with either 1 μM RA or RA plus dibutyryl cAMP (250 μM) and theophylline (500 μM) (RACT) for 96 h, the F9-Wt cell line develops a differentiation phenotype characterized by the development of cellular processes, distinct cell borders, and irregular cell shape. In contrast, in the RAR$\gamma^{-/-}$ line this differentiation phenotype is much less pronounced. Thus, the absence of RARγ attenuates the changes in morphology associated with RA-induced differentiation. (*a*) F9-Wt. (*b*) F9-Wt + RA, 96 h. (*c*) F9-Wt + RACT, 96 h. (*d*) F9-Wt + CT, 96 h. (*a′*) F9-Wt-γ-119-14. (*b′*) F9-Wt-γ-119-14 + RA, 96 h. (*c′*) F9-Wt-γ-119-14 + RACT, 96 h. (*d′*) F9-Wt-γ-119-14 + CT, 96 h.

Hoxb-1 and CRABPII RNAs are induced to the same level as in wt cells, but at 48 h after RA treatment these RNAs do not decrease as rapidly as those in the wt cells.

Not all differentiation-related genes are affected in the RAR$\gamma^{-/-}$ lines. For instance, the RA responsive genes Rex-1 and SPARC are regulated in the RAR$\gamma^{-/-}$ lines as they are in wt cells (Fig. 3). Interestingly, expression of RARα and RARβ (Fig. 3), as well as that of RXRα and RXRβ (data not shown), remains unaltered in the RAR$\gamma^{-/-}$ lines. These results suggest that RARγ plays a specific role in the RA regulation of the "early" RA-inducible gene *Hoxa-1*, as well as in that of the "late" RA-inducible laminin B1 and collagen type IV (α1) genes.

A gel mobility-shift assay was used with the addition of monoclonal antibodies specific for RARα, -β, and -γ to investigate which RARs are present in the wt and RAR$\gamma^{-/-}$ F9 cells before and after RA treatment. With extracts of untreated F9-Wt cells, specific complexes are observed using RARα and RARγ but not RARβ antibodies, indicating the presence of both RARα and RARγ but not RARβ in these cells (Fig. 4, lanes 1–3 and 7–9). Interestingly, the intensity of the complex formed with RARγ is stronger than that of the RARα complex, suggesting that F9-Wt cells are richer in RARγ than in RARα. In contrast, and as expected, no RARγ complex is detected in RAR$\gamma^{-/-}$ cells under conditions where the intensity of the RARα complex is similar to that of wt cells (compare lanes 1 and 7). Control transfection experiments with vectors expressing either RARα, -β, or -γ indicate clearly that the absence of RARγ in the "mutant" cells is due to the disruption of the RARγ alleles (lanes 12–15 and 22–24). After RA treatment, RARβ complexes are similarly "induced" in wt and RAR$\gamma^{-/-}$ cells (compare lanes 4–6 and 10–12), in agreement with the Northern blot data above for RARβ. As expected, the RA treatment does not affect the formation of RARγ complexes, which remain absent in the RAR$\gamma^{-/-}$ cells (lane 12).

Taken together the results described above indicate that RARγ is absent in the mutant cells, whereas RARβ is induced as in wt cells. Since the RAREs of the *Hoxa-1* and RARβ genes appear to be identical (23, 24), this latter result indicates (*i*) that RARβ induction is autoregulatory, or (*ii*) that due to their different promoter context (29) the RAREs of the RARβ and *Hoxa-1* are bound and activated by different receptors (e.g., RARγ in the case of *Hoxa-1* and RARα in the case of RARβ), and/or (*iii*) that there exists a greater functional redundancy among RARs in the case of the RARβ promoter than in the case of *Hoxa-1* (e.g., the RARβ promoter could be stimulated by both RARα and RARγ, whereas the *Hoxa-1* promoter would be preferentially stimulated by RARγ).

DISCUSSION

We show here that the inactivation of RARγ in F9 stem cells prevents several aspects of the RA-associated differentiation pathway observed in F9-Wt cells. RARγ gene disruption specifically results in a drastically reduced induction of Hoxa-1 RNA and in the lack of induction of the laminin B1 and collagen type IV (α1) RNAs. In contrast, *Hoxb-1* and CRABPII show normal RA induction in the RAR$\gamma^{-/-}$ lines but are not correctly down regulated. RARβ, SPARC, and Rex-1 regulation are unaffected by the lack of RARγ. This work represents identification of some of the direct and/or indirect gene targets of RARγ. In the developing mouse, RARα, RARβ, and RARγ are expressed in distinct embryonic regions, suggesting that each RAR performs a specific developmental function (12, 13). Our observations support the idea that each RAR regulates the expression of specific genes. We have also shown that RARγ is involved in the morphological changes associated with RA-induced F9 stem

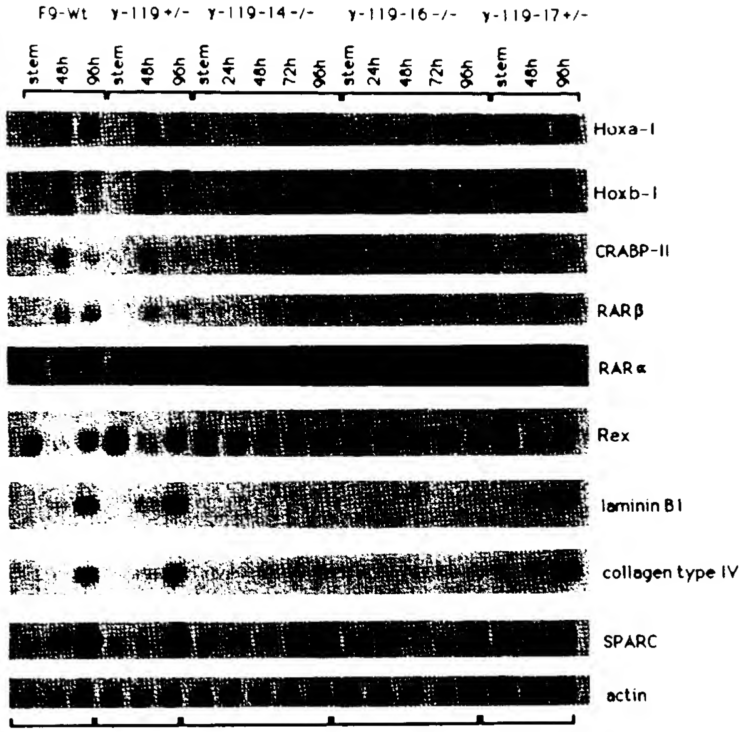

FIG. 3. The absence of RARγ RNA results in a reduction of RA-induced Hoxa-1, laminin B1, and collagen type IV (α1) RNA expression. The ability of F9 stem cells lacking the RARγ gene to respond to RA-induced differentiation was tested by Northern blot analysis. The RAR$\gamma^{-/-}$ cell lines F9-Wt-γ-119-14 and F9-Wt-γ-119-16 exhibit reduced levels of the Hoxa-1 RNA and do not express detectable levels of laminin B1 or collagen type IV (α1) RNAs compared to the F9-Wt and RAR$\gamma^{+/-}$ cell lines F9-Wt-γ-119 and F9-Wt-γ-119-17. Hoxb-1 and CRABPII RNA levels are induced to the same level as in the wt line; however, they do not decrease as rapidly as in wt cells. Expression of RARα, RARβ, Rex-1, and SPARC RNAs is not significantly affected in the RAR$\gamma^{-/-}$ lines. This experiment was performed twice with similar results.

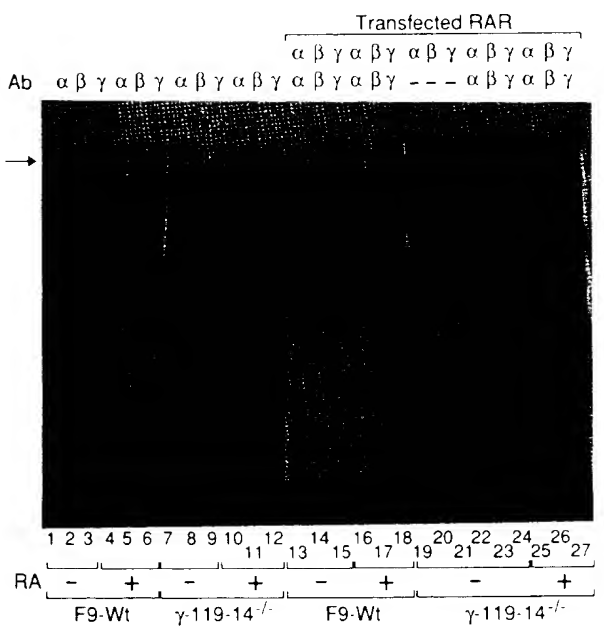

FIG. 4. Lack of binding of RARγ to the Hoxa-1 RARE in a RAR$\gamma^{-/-}$ line. Binding of the RARs present in extracts of F9-Wt and RAR$\gamma^{-/-}$ cell lines (with or without RA treatment) was investigated by a gel mobility-shift assay. A radiolabeled oligonucleotide representing the Hoxa-1/RARβ RARE was incubated with nuclear extracts prepared from F9-Wt cells (lanes 1–3, RA untreated; lanes 4–6, RA treated), F9-Wt-γ-119-14 RAR$\gamma^{-/-}$ cells (lanes 7–9, RA untreated; lanes 10–12, RA treated), F9-Wt cells (lanes 13–15, RA untreated; lanes 6–18, RA treated), and F9-Wt-γ-119-14 RAR$\gamma^{-/-}$ cells (lanes 19–24, RA untreated; lanes 25–27, RA treated) transfected with murine RARα (lanes 13, 16, 19, 22, and 25), RARβ (lanes 14, 17, 20, 23, and 26), or RARγ (lanes 15, 18, 21, 24, and 27) expression vectors. Arrow indicates the shifted complex formed in the presence of mouse monoclonal antibodies Ab9α(F) (lanes 1, 4, 7, 10, 13, 16, 22, and 25), Ab8β(F)2 (lanes 2, 5, 8, 11, 14, 17, 23, and 26), and Abγ(mF) (lanes 3, 6, 9, 12, 15, 18, 24, and 27) specifically directed against the F region of RARα, -β, and -γ, respectively.

cell differentiation. This abnormal morphological differentiation of RAR$\gamma^{-/-}$ cells may result in part from reduced *Hoxa-1* expression, as we have previously demonstrated that ectopic expression of the *Hoxa-1* gene in the absence of RA leads to a morphological change, which is, however, clearly distinct from that induced by RA (30). In this respect, we note that *Hoxa-1* expression does not induce the morphological differentiation of embryonal carcinoma P19 cells (31).

Disruption of the RARγ gene in mice does not produce the alterations associated with *Hoxa-1* disruption (14, 32, 33). There may be several explanations for this apparent discrepancy. It is possible that the control of *Hoxa-1* expression in the context of the whole animal is different from that in a tissue culture model or that the functional redundancy among the three RAR types is greater in the developing mouse, providing a means for compensating for the loss of one receptor. Note that RARα but not RARγ transcripts were detected in the rhombencephalic region that is affected by *Hoxa-1* disruption (34). The possibility of functional redundancy among the three RARs *in vivo* is supported by the results of additional RAR disruptions in the mouse. Mice lacking the RARα1 isoform possess no developmental or growth abnormalities (35, 36), suggesting that the loss of RARα1 can be compensated for during development. Indeed, even in the F9 RAR$\gamma^{-/-}$ cells, there appears to be some level of redundancy, since Hoxa-1 is detectable at low levels during the latter phase of RA treatment. The low levels of *Hoxa-1* expression at late times of RA treatment either may result from RARα action or may be the result of the increase in RARβ expression at later times. Further studies with F9 embryonal carcinoma cells disrupted for the RARα and/or RARβ genes should be useful in resolving these questions of functional redundancies among RARs.

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Modulation of gene expression in human osteoblasts by targeting a distal promoter region of human estrogen receptor-α gene

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Abstract

Estrogen receptor (ER) α is expressed during osteoblast differentiation; however, both its functional role in bone metabolism and its involvement in osteoporotic pathogenesis caused by estrogen deficiency are not well understood. Loss of ERα gene expression could be one of the mechanisms leading to osteoporosis. Therefore, we investigated a possible modulation of ERα gene expression in a human osteoblastic cell line and in four primary osteoblast cultures by using a decoy strategy. Double stranded DNA molecules, mimicking a regulatory region of the ERα gene promoter (DNA-102) and acting as a 'silencer' in breast cancer cells, were introduced into osteoblasts as 'decoy' cis-elements to bind and functionally inactivate a putative negative transcription factor, and thus to induce ERα gene expression.

We found that the DNA-102 molecule was able to specifically bind osteoblast nuclear proteins.

Before decoy treatment, absence or variable low levels of ERα RNAs in the different cultures were detected. When the cells were transfected with the DNA-102 decoy, an increase in expression of ERα and osteoblastic markers, such as osteopontin, was observed, indicating a more differentiated osteoblastic phenotype both in the cell line and in primary cultures. These results showed that the DNA-102 sequence competes with endogenous specific negative transcription factors that may be critical for a decrease in or lack of ERα gene transcription. Therefore, osteoblastic transfection with the DNA-102 decoy molecule may be considered a tempting model in a putative therapeutic approach for those pathologies, such as osteoporosis, in which the decrease or loss of ERα expression plays a critical role in bone function.

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Introduction

Estrogens regulate a variety of metabolic processes through their specific nuclear receptors, which belong to a nuclear receptor superfamily (Carson-Jurica *et al.* 1990, Mangelsdorf *et al.* 1995) and act as ligand-dependent transcription factors (Green & Chambon 1988). Two specific estrogen receptors have been identified: ERα (Green *et al.* 1986, Auchus & Fuqua 1994) and, more recently, ERβ (Mosselman *et al.* 1996, Vidal *et al.* 1999). Both receptors exibit a specific tissue distribution and modulate activities of different estrogen responsive gene promoters in a different manner (Bord *et al.* 2001, Braidman *et al.* 2001).

The importance of estrogen and nuclear ERs to skeletal growth and bone metabolism is supported by a body of evidence (Eriksen *et al.* 1988, Bodine *et al.* 1998, Rickard *et al.* 1999, Compston 2001). Nevertheless, the network of interactions and molecular mechanisms is very complex and the design of a unique model of estrogen action in bone is very difficult. Recent advances have defined potential sites of estrogen action within the bone microenvironment: these mainly include proliferation and differentiation of osteoprogenitor cells, activity of mature osteoblasts and osteoclasts, bone matrix synthesis and bone resorption, and interaction with co-regulatory factors (Rickard *et al.* 1999, Spelsberg *et al.* 1999).

Expression studies in skeletal cells both *in vitro* and *in vivo* have demonstrated that the concentration of ERα is higher than ERβ in bone and in osteoblasts at all stages of differentiation (Arts *et al.* 1997, Denger *et al.* 2001).

As for the clinical aspects, the positive effect of estrogens on bone homeostasis is well known. Estrogen replacement therapy reduces the incidence and severity of pathologies such as osteoporosis and cardiovascular disease in postmenopausal women (Pacifici 1996, Riggs 2000), even if long term estrogen treatment increases the risk of endometrial and breast cancers. At present, a number of studies

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aimed at understanding the wide spectrum of effects exerted by estrogen on the bone have described the development of drugs and therapeutic approaches for the treatment of osteopenic disorders (Windahl *et al.* 1999, Rodan & Martin 2000, Compston 2001), such as osteoporosis, tumor-associated osteolysis, rheumathoid arthritis, periodontal disease and orthopedic implant osteolysis.

Since the expression level of endogenous ERs is limiting for estrogen responses, it is tempting to speculate that a strategy able to modulate ERs gene expression may be a new tool for stimulating bone formation. An increase in gene expression could be fulfilled either by recruiting positive transcription factors or by reducing the action of negative factors. We focused our attention on ERα gene expression and we hypothesized that subtracting negative transcription factors able to bind ERα gene promoter(s), by using specific approaches or agents, could result in a reduction in their negative effect and an increase in ERα gene expression.

The concept of using nucleic acids to bind target proteins has been explored as a way of manipulating gene expression in living cells (Mann & Dzau 2000). This strategy involves the delivery of double-stranded DNA molecules termed 'decoys' which are able to squelch the activity of the target transcription factor (Piva & Gambari 1999). The competition for trans-acting factors between the endogenous cis-elements present on the target gene and the exogenously added decoy DNA molecule, containing a sequence identical to that of the specific cis-element, results in an inhibition or attenuation of the 'authentic' interaction of trans-factor(s) with its cis-element(s). Therefore, this approach represents a method for testing the biological involvement of genomic sequences in the regulation of gene expression and in the maintenance of a specific phenotype (Morishita *et al.* 1996, Sharma *et al.* 1996, Yamashita *et al.* 1998, Wang *et al.* 2000). Additionally, this approach can be considered a useful method for modulating the gene expression for potential therapeutic intervention (Morishita *et al.* 1995, Tomita *et al.* 1999, Mann & Dzau 2000).

In this study, the involvement of the ERα in bone cells was investigated by transfecting cultured human osteoblast cells with a decoy molecule against a distal promoter of the ERα gene. The decoy molecule that we propose is a synthetic double-stranded DNA belonging to the P3 distal promoter of the ERα gene (−3258/−3157, termed DNA-102), showing a high affinity for a putative negative transcription factor (nTF) found in ER-negative cells. In a previous study, we transfected this sequence into ER-negative breast cancer cells, and we obtained the reactivation of ERα gene transcription (Penolazzi *et al.* 2000).

The aim of this study was to investigate the ability of DNA-102 decoy to bind to nTF and to affect the induction of ERα gene expression in the TE85 osteosarcoma cell line and in human primary osteoblasts. The analysis concerns ERα because levels of ERβ mRNA were undetectable in the primary osteoblast cultures analyzed.

We report that, in these osteoblast-like cells, the DNA-102 decoy increases both the ERα gene expression, in particular through the activity of upstream ERα gene promoters, and the expression of osteopontin (OPN) (Denhardt & Guo 1993) and osteonectin (ON) (Termine *et al.* 1981) that are typical markers of osteoblastic function and differentiation. By contrast, ERβ gene expression was not reactivated by decoy treatment.

Therefore, our experimental approach may contribute to the development of bone anabolic therapeutic molecules that would stimulate bone formation through the specific manipulation of gene expression.

Materials and Methods

Materials

Egg phosphatidyl choline was purchased from Lipid Products (Nutfield Nurseries, Surrey, UK). The cationic surfactant N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium methyl-sulfate (DOTAP) was purchased from Sigma Chemical Co. (St. Louis, MO, USA)

As decoy molecule, a DNA fragment belonging to the 5′ region of the human (h) estrogen receptor gene, 102 bp in size (DNA-102), was generated by PCR using RA1 (5′-GCCATTGTTGACCTACAGGAG-3′) and RA4 (5′-TATTTATATCCAGTATTTATTTTCAATACTGACT-3′) primers. As control, a 150 bp plasmidic fragment (DNA-150) was used. pBLCAT8 ERCAT1 (Piva *et al.* 2000) and pGEX-2TK (Nilsson *et al.* 1985) recombinant plasmids were used as templates for DNA-102 and DNA-150 respectively. After amplification, DNAs were purified by an ultrafiltration procedure with the Microcon-30 system (Amicon, Inc, Beverly, MA, USA) as previously described (Penolazzi *et al.* 1997).

Liposome preparation

Cationic liposomes, composed of egg phosphatidyl choline (PC) and the cationic surfactant DOTAP (PC:DOTAP, 8:1 mol/mol), were prepared by reverse phase evaporation followed by three extrusion cycles through 200 nm pore size polycarbonate membranes. The extrusion step was performed in order to obtain unilamellar liposomes with a homogeneous size distribution, as confirmed by freeze-fracture electron microphotographs (Cortesi *et al.* 1996).

Cell culture and DNA transfection efficiency

Normal human spongy bone specimens were collected during surgical procedures. Patients were in good health consistent with their age, and were not suffering from

autoimmune or metabolic diseases or malignancies. They were affected by arthritis of the hip and in the case of patients 3 and 4 clinical diagnosis of osteoporosis was made. For the surgical procedure we followed Hardinge's surgical approach to the hip. As regards the ethics of the experimental procedures on human subjects, informed consent was obtained from each patient after full explanation of the purpose and nature of all procedures used.

Bone specimens were cultured according to Maurizi *et al.* (1983). Primary cultures were grown in Falcon flasks containing Eagle's Minimum Essential Medium supplemented with 20% fetal bovine serum (Gibco, Gaithersburg, MD, USA) and antibiotics at 37 °C in an humidified atmosphere of 5% CO_2 (Sollazzo *et al.* 1997). Subcultures were obtained about 30 days later. The TE85 osteosarcoma cell line was grown in the same conditions.

Decoy DNA molecule (600 ng) was used to transfect cells at 60% confluence plated in 31-mm diameter plates. DNA was mixed with cationic liposome suspension (lipid:DNA ratio 10:1 w/w) in a final volume of 200 µl. After 30-min incubation at room temperature, 200 µl serum-free medium were added to the liposome/DNA complex and used to transfect one well. Twenty-four hours later, the transfection solution was replaced with complete medium that was not deprived of endogenous estrogenic activity. After transfection, cells were washed five times with PBS.

Alkaline phosphatase activity

Alkaline phosphatase (ALP) activity was measured in confluent human osteoblastic cells by the hydrolysis of *p*-nitrophenylphosphate (PNPP) according to Ibbotson *et al.* (1986). Enzyme activity was expressed as U/mg protein. One unit was defined as the amount of enzyme which hydrolysed 1 µmol PNPP/minute. Cell protein was determined according to the Lowry method (Lowry *et al.* 1951). The effect of 1,25-dihydroxyvitamin D3 (1,25-$(OH)_2D_3$) on ALP activity was verified after incubation in medium containing 10 nM 1,25-$(OH)_2D_3$ for 48 h.

Analysis of gene transcription

Gene expression was detected by reverse transcription-polymerase chain reaction (RT-PCR) on total RNA (1–5 µg) from transfected cells.

The amplification reactions were performed using the SuperScript One-Step RT-PCR System (Life Technologies, GibcoBRL, Gaithersburg, MD, USA) and a Violet Thermal Cycler. The following primers and conditions were used. ERα: forward (F) = 5′-CTATATG TGTCCAGCCACCAACC-3′ (exon 3), reverse (R) = 5′-CTCTACACATTTTCCCTGGTTCCT-3′ (exon 6); 30 cycles: 60 s at 94 °C, 60 s at 57 °C and 60 s at 72 °C. ERβ: F = 5′-ATCTTTGACATGCTCCTGGC-3′, R = 5′-ACGCTTCAGCTTGTGACCTC-3′; 30 cycles: 60 s at 94 °C, 60 s at 56 °C and 60 s at 72 °C. OPN: F = 5′-CAGAATCTCCTAGCCCCACA-3′, R = 5′-AA CTCCTCGCTTTCCATGTG-3′; 30 cycles: 60 s at 94 °C, 60 s at 51 °C and 60 s at 72 °C. ON: F = 5′-GTATCTGTGGGAGCTAATCCT-3′, R = 5′-AGAGT CGAAGGTCTTGTTGTC-3′; 30 cycles: 60 s at 94 °C, 60 s at 52 °C and 60 s at 72 °C. β-Actin: F = 5′-TGACGGGGTCACCCACACTGTGCCCATCTA-3′, R = 5′-CTAGAAGCATTTGCGGTGGACGATGGAG GG-3′; 20 cycles: 45 s at 94 °C, 45 s at 60 °C and 45 s at 72 °C.

PCR amplifications specific for different ERα transcripts were performed under the following conditions. FG/R2: FG = 5′-TCGTCCTGGGAGCTGCACTT-3′, R2 = 5′-GATAATCGACGCCAGGGTGGCAGA-3′; 30 cycles: 60 s at 94 °C, 60 s at 53 °C and 60 s at 72 °C. FP/R1: FP = 5′-AAGACGTTCTTGATCCAGC-3′, R1 = 5′-ACCAAAGCATCTGGGATG-3′; 30 cycles: 60 s at 94 °C, 60 s at 54 °C and 60 s at 72 °C. FH/R1: FH = 5′-AGGAAGGAGTAAGCACAAAG-3′, R1 = 5′-ACCAAAGCATCTGGGATG-3′; 30 cycles: 60 s at 94 °C, 60 s at 48 °C and 60 s at 72 °C.

All amplifications were compared with a negative control (primers without RNA) and the levels of expression of the different genes were normalized against the β-actin mRNA content using a densitometric analysis. RT-PCR products were separated on agarose gel, electrophoresed and, for ER RNA analysis, were subsequently blotted onto nylon membrane using standard procedures (Penolazzi *et al.* 1998). Hybridizations were performed with the following ^{32}P-labeled probes: pOR15 (Green *et al.* 1986) for total ERα transcription analysis, pGHER1 (Ponglikitmongkol *et al.* 1988) for FG/R2, FP/R1 and FH/R1 PCR amplifications used to discriminate the activity of different promoters, and pSG5-hERβ (Ogawa *et al.* 1998) for ERβ mRNA.

Western blot analysis

Cell extracts from TE85 cells were separated by 10% SDS-PAGE, essentially according to Laemmli (1970), and proteins were then transferred to nitrocellulose membrane (Hybond C). After electroblotting, proteins were visualized using Ponceau S reagent (Sigma). The blots were blocked for 2 h at room temperature with 1 × phosphate-buffered saline containing 0·1% Tween 20 (PBST) and 3% BSA, incubated for 2 h with purified monoclonal antibody (290 ng/ml) to the human ERα (H222, diluted 1:1000), polyclonal antisera against the human bone OPN (LF-123, diluted 1:1000) and bovine bone osteonectin (BON-1, diluted 1:1000) in blocking solution. The blots were then washed three times with PBST for 30 min and incubated for 45 min with PBST containing alkaline phosphatase-conjugated goat anti-rat IgG antibody (Promega) diluted 1:4000, and washed three

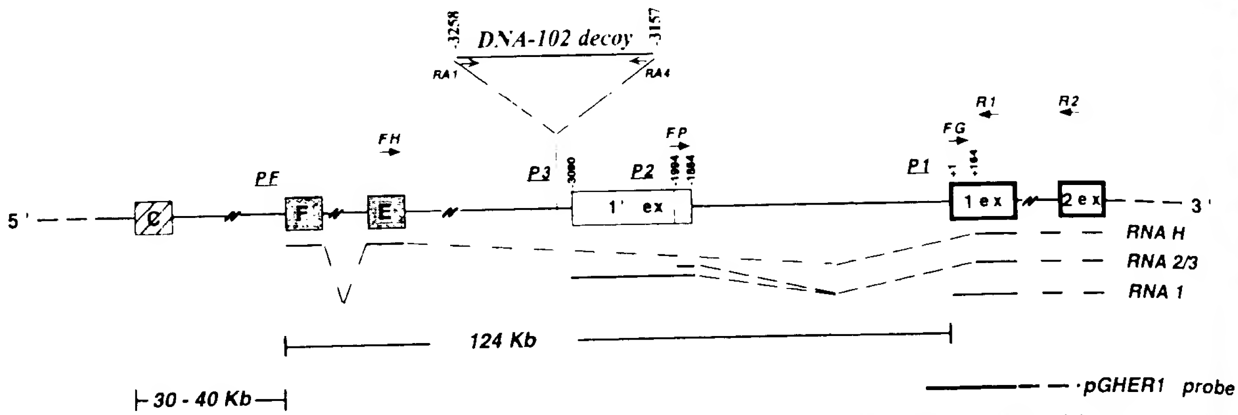


Figure 1 Schematic representation of the 5′ upstream region of the human estrogen receptor (ER)α gene and some of the corresponding transcripts so far characterized. The exons are indicated with boxes and the P1 canonical promoter, P2, P3 and PF distal promoters are shown. Exon usage and alternative splicing patterns for some of the different RNA isoforms are schematically represented. Nucleotide +1 represents the transcription start site of the canonical ERα RNA (RNA1). Upstream transcription start sites at −1994 and at −3090 for isoforms 2 and 3 respectively, as well as the splicing acceptor site position at +164 inside exon 1 are indicated. RNA H indicates the RNA transcript containing the E and F upstream exons. The location of the DNA-102 decoy molecule, primers employed for the RT-PCR experiments and pGHER1 probe are also shown.

times with PBST for 30 min. Immunoreactive proteins were visualized using ProtoBlot Western Blot AP Systems (Promega).

Electrophoretic mobility shift assay

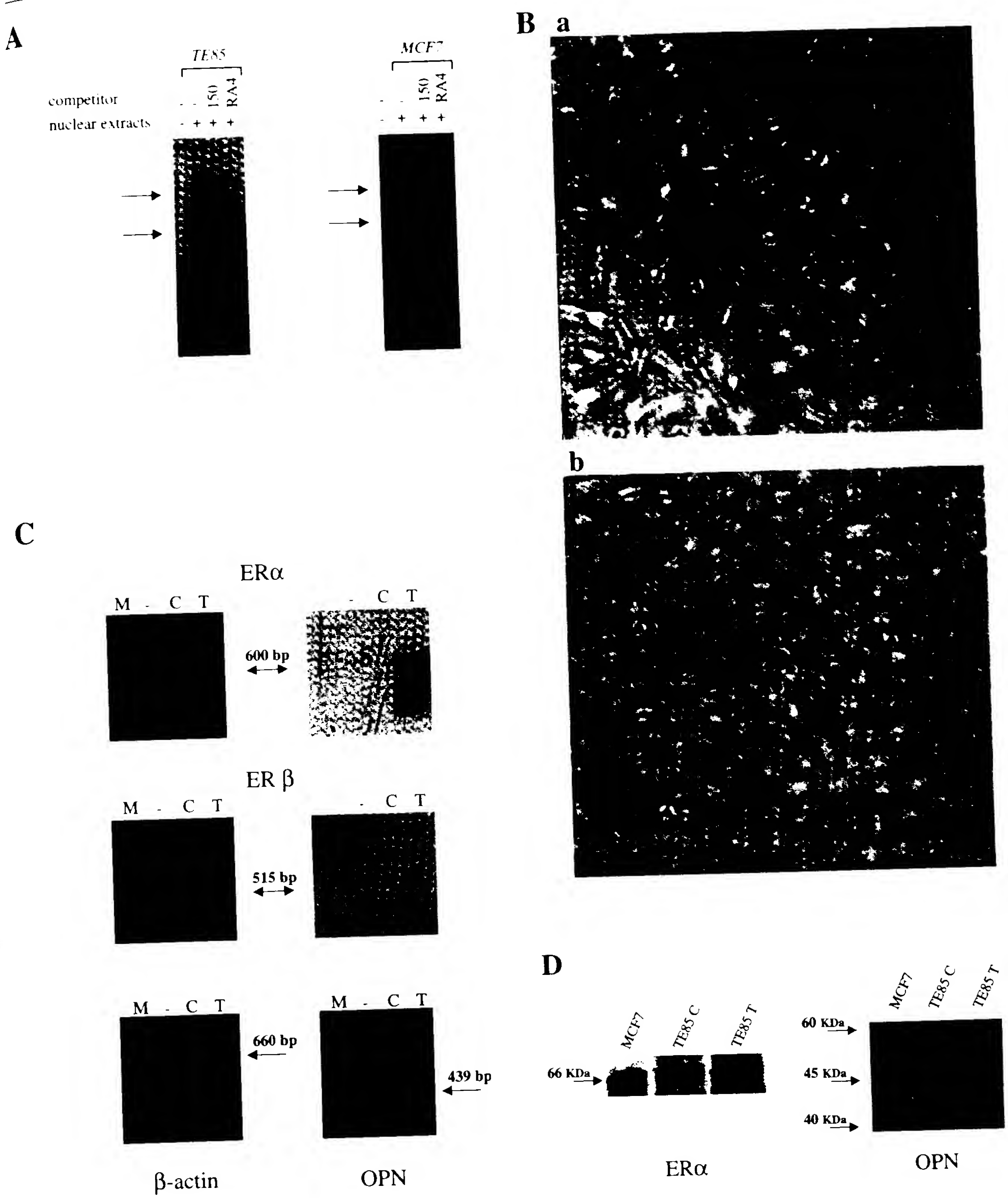
RA4 (−3190/−3157) radiolabeled oligonucleotides inside DNA-102 were used as a probe in the incubation with nuclear extracts from TE85 and MCF7 cells. To prepare nuclear extracts, cultured cells were washed twice with PBS and collected with a scraper. The cytoplasmic membranes were ruptured mechanically using a Dounce B homogenizer, and nuclear proteins were obtained essentially as described by Dignam *et al.* (1983). Protein concentration was determined using a Bio-Rad (Hercules, CA, USA) protein assay. Nuclear extracts were incubated with 0·1 ng (6000 c.p.m.) labeled probes in 1 × binding buffer (10 mM Tris–HCl pH 7·5, 20 mM KCl, 10 mM $MgCl_2$, 10 mM dithiothreitol, 5 mM EDTA, 0·01% Triton X-100, 0·5% glycerol) containing 1·2 mg poly(dI-dC).poly(dI-dC) (Sigma) for 30 min at room temperature. Specific competitors including unlabeled probes and nonspecific competitor (150 bp PCR product from pGEX-2TK plasmid) were added at different molar excesses. The DNA–protein complexes were separated from the uncomplexed DNA on 6% polyacrylamide gel in 0·25 × Tris–borate-EDTA by electrophoresis at 150 volts. Gels were dried and then exposed to X-ray film.

Results

DNA-102 molecule interacts with nuclear extracts from osteoblasts

In order to look for nuclear proteins that might bind to the DNA-102 sequence to be used as the decoy molecule, we tested the RA4 oligonucleotide in the electrophoretic mobility shift assay with nuclear extracts from the TE85 osteoblastic-like cell line. This oligonucleotide covers the sequence from −3190 to −3157, inside the DNA-102

Figure 2 (A) Gel mobility shift assay. RA4 (−3190/−3157) radiolabeled oligonucleotide belonging to the ERα P3 promoter was used as a probe in the incubation with nuclear extracts from TE85 osteosarcoma and MCF7 breast cancer cells. Specific competitors including unlabeled probe and nonspecific competitor (150 bp PCR product from pGEX-2TK plasmid) were added at different molar excess. The retarded bands generated by specific interaction between oligonucleotides and nuclear factors are arrowed. (B) Photomicrographs of TE85 cells treated with the DNA-102 decoy molecule complexed with cationic liposomes (a), or left untreated (b). Original magnification: × 100. (C) Effect of DNA-102 decoy on modulation of ERα, ERβ and osteopontin (OPN) gene expression. Expression of ERα, ERβ, OPN and β-actin mRNA was detected by RT-PCR on total RNA from decoy-treated (T) or untreated (C) TE85 cells. All amplifications were compared with a negative control (primers without RNA) and the levels of ERα and OPN mRNA were normalized against the β-actin mRNA content using a densitometric analysis. In the case of ERα mRNA, the specificity of electrophoretic data was confirmed by hybridization with a ^{32}P-labeled ERα specific probe (pOR15) followed by autoradiography. For ERβ mRNA, the specific probe used was pSG5-hERβ (Ogawa *et al.* 1998). The specific RT-PCR products are arrowed. M, molecular weight marker (HaeIII restricted pBR322 DNA); (−), negative control. (D) Determination of ERα and OPN by immunoblot analysis. Twenty micrograms whole cell extracts from TE85 cells treated with the DNA-102 decoy molecule (TE85 T) or untreated (TE85 C) were resolved by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose filter; blots were probed with ERα-specific monoclonal antibody and OPN-specific polyclonal antibody respectively. MCF7 cellular extracts were used as a control.


A
TE85
MCF7
competitor - - 150 RA4
nuclear extracts - + + +
B a
b
C
ERα
M - C T
600 bp
- C T
ER β
515 bp
660 bp
439 bp
β-actin
OPN
D
MCF7
TE85 C
TE85 T
66 KDa
60 KDa
45 KDa
40 KDa
ERα
OPN

molecule (see Fig. 1), and it was previously characterized as the sequence mainly involved in DNA–protein interactions (Penolazzi et al. 2000).

As shown in Fig. 2A, two main labeled complexes were generated by the binding of nucleoproteins to the RA4 oligonucleotide, with a pattern which was very similar to that observed when nuclear extracts from MCF7 ER-positive breast cancer cells were used. The specific DNA–protein complexes were displaced by a 250-fold excess of unlabeled homologous competitor, but not by the unlabeled unrelated 150 bp double stranded (ds)-DNA, demonstrating the specificity of the observed protein–DNA interactions. Therefore, these findings suggest that the osteoblastic-like cells express nuclear protein(s), that are able to bind specifically to the DNA-102 sequence.

The decoy effect on gene expression in the TE85 cell line

The PCR product DNA-102, including the RA4 oligonucleotide, was used as decoy molecule because it retains a major nuclease resistance compared with the RA4 oligonucleotide. Transfection experiments were carried out in medium not deprived of endogenous estrogenic activity, in the presence of DNA-102 complexed with PC:DOTAP cationic liposomes (Cortesi et al. 1996). Cytotoxic effects were not observed: decoy-treated cells showed only slight changes in their morphology (see Fig. 2B); in addition, their ability to proliferate, in comparison with untreated cells, was only slightly decreased (data not shown).

Reverse transcription-PCR (RT-PCR) analysis was then performed in order to investigate whether the decoy treatment would affect gene expression in osteoblastic cells. Total RNA was isolated from treated and untreated cells, transfected in duplicate; subsequently, the same amount of RNA was reverse transcribed with random and ERα-specific oligonucleotides. The cDNA obtained was subjected to the PCR amplification in a first step for ERα gene using primers specific for exon 3 and exon 6, and then for the osteopontin (OPN) gene, to evaluate the effect of decoy DNA-102 on estrogen-related gene expression. The cDNA was also amplified with primers specific for a gene whose expression is not estrogen dependent, such as β-actin. In all the experiments, the β-actin PCR product was used as an internal control in order to obtain a semiquantitative comparison of the gene expression. A representative experiment is shown in Fig. 2C. The levels of PCR product derived from the ERα transcript were almost undetectable in the TE85 cell line, but when the cells were transfected with the DNA-102 decoy molecule, the PCR product was clearly evident, as seen from agarose gel analysis and confirmed by hybridization with ^{32}P-labeled pOR15 specific probe. Also, in the case of OPN mRNA, decoy treatment resulted in a positive effect. The mean values obtained by densitometric analysis of the band intensity in different RT-PCR experiments, expressed as optical density (O.D.) arbitrary units, were 0·38 untreated cells 1·43 treated cells for ERα, 0·195 untreated cells 0·824 treated cells for OPN and 1·510 untreated cells 1·383 treated cells for β-actin. By contrast, ERβ mRNA was undetectable even after DNA-102 decoy treatment. When the cells were transfected with an unrelated plasmid 150 bp PCR product, the expression of ERα and β-actin genes was completely unaffected (data not shown), thus demonstrating that the effect of DNA-102 decoy can be considered specific. Therefore, these experiments showed that a specific gene expression may be positively regulated by DNA-102 decoy in the osteoblastic cell line analyzed.

Next, we analyzed the promoter usage in the TE85 cell line and the effect of the decoy on the upstream ERα transcripts, but, as expected from the very low level of ERα gene transcription, this analysis was not satisfactory.

An examination of protein levels of ERα and osteopontin by Western blot analysis was then carried out. As shown in Fig. 2D, these cells were found to express the marker of the osteoblast phenotype (OPN) typically in the three isoforms (60, 45 and 40 kDa) and the ERα protein. After the decoy treatment, an increase in ERα and OPN level was observed.

The decoy effect on gene expression in human primary osteoblasts

Next, we examined the ability of decoy DNA-102 to induce ERα gene expression in primary osteoblasts. These human bone-derived cells displayed specific osteoblast features such as the expression of high ALP activity that increased after 1,25-$(OH)_2D_3$ treatment (Beresdorf et al. 1986), as shown in Table 1.

Also, in the case of these cells, the transfection experiments were carried out in medium not deprived of endogenous estrogenic activity, with DNA-102 complexed with PC:DOTAP cationic liposomes. During the decoy treatment cytotoxic effects were very slight, as confirmed by the absence of significant cellular morphological changes (Fig. 3A).

The cDNAs obtained from untreated and decoy-treated cells were amplified by PCR, first using primers specific for exon 3 and exon 6 of the ERα gene to estimate its expression as a whole, and then using the appropriate primers to distinguish transcription at upstream or at main promoters (see Fig. 1 for the localization of the primers). When different RNA isoforms of a gene originate from upstream exonic sequences and from alternative splicing events, such as in the case of the ERα gene, it is possible to analyze the level of expression of a single isoform by RT-PCR choosing the forward primer inside the specific upstream exon used. The primers for the amplification of the only canonical ERα transcript were: the forward FG (+20/+39), inside exon 1 and located upstream of the splice site position at +164 that is employed as a splicing

Table 1 Alkaline phosphatase (ALP) activity (basal and following 1,25-$(OH)_2D_3$ stimulation) in human primary osteoblasts, expressed as U/mg protein × 10^3. Values are the means ± s.d. of three parallel measurements

Patient no.	Sex	Age (years)	ALP basal	ALP 1,25-$(OH)_2D_3$
1	Female	65	4·5 ± 0·17	5·4 ± 0·1
2	Male	64	15·07 ± 0·21	19·9 ± 0·96
3	Female	78	53 ± 1	64·07 ± 0·67
4	Female	80	11 ± 0·5	16 ± 0·53

acceptor site of the other ER RNA isoforms analyzed; the reverse R2 (+695/+718), inside exon 2. The forward primers used for the amplification of some possible upstream transcripts were: FP, inside exon 1 (Keaveney *et al.* 1992, Piva *et al.* 1993) and FH, inside exon E described by Flouriot *et al.* (1998) and corresponding to exon Hb described by Thompson *et al.* (1997). R1 primer (+251/+268) localized inside exon 1 of the ERα gene was used as reverse primer. To improve gene expression analysis, the levels of type 1 (FG/R2), type 2/3 (FP/R1) and type H (FH/R1) ERα mRNAs were estimated by RT-PCR followed by specific hybridization with upstream pGHER1 probe (Ponglikitmongkol *et al.* 1988) as shown in Fig. 3B, in which a representative experiment, corresponding to sample no. 1, is illustrated. The levels of ERβ, OPN and ON gene expression were also evaluated using specific primers (Fig. 3B). All RT-PCR products corresponding to endogenous and decoy-dependent gene expression levels of each sample were then subjected to densitometric analysis: the results are summarized in Table 2 as total ERα mRNA expression level, and in Table 3 as OPN and ON mRNA levels.

The highest level of ERα RNA was observed in the cell culture obtained from the youngest female patient, sample no. 1 (aged 65 years) (Table 2 and Fig. 3B). As regards promoter usage, the canonic P1 promoter was active only in sample 1 because only in this patient was the ERα mRNA 1 (FG/R2) detected (see Fig. 3B). The P2/P3 and PF distal promoters were preferentially used in the osteoblasts from samples 2, 3 and 4 (see Table 2): in these cases the ERα mRNA 1 (FG/R2) was not detected, whereas the ERα mRNA type 2/3 (FP/R1) and especially the ERα mRNA type H (FH/R1) were detected.

The usage of each specific promoter did not change when the cells were transfected with DNA-102 decoy molecule, suggesting that the ERα gene regulatory regions, that are specifically used in bone cells, play a critical role in the committed differentiation of osteoblasts. After decoy treatment, however, the level of ERα gene transcription was increased in three of the four samples analyzed. The major increase in transcriptional levels was obtained for sample 1, whereas in sample 4, corresponding to the osteoblasts of the oldest patient (age 80 years), ERα gene transcription seemed to be unaffected by the decoy treatment (see Table 2).

As far as the expression of markers of osteoblastic differentiation was concerned, the DNA-102 decoy treatment resulted in a marked increase in OPN RNA expression in three of the four cultures (nos 1, 2 and 4), and in a slight increase in ON RNA expression levels (see Table 3). By contrast, ERβ mRNA levels were undetectable even after decoy treatment in all primary osteoblast cultures analyzed.

Discussion

Many studies employing Northern blot, RT-PCR and immunohistochemical analysis indicate that the level of estrogen receptors in different osteoblastic cells is very low, in spite of their being highly responsive to estrogens, and that there is a heterogeneity of ERα and ERβ expression among osteoblastic cells (Ikegami *et al.* 1993, 1994, Rao & Murray 2000, Bord *et al.* 2001, Compston 2001).

The questions remain as to what level of ERs expression is sufficient to sensitize osteoblasts to estrogen and if the possibility to modulate ERs gene expression may be a tool to stimulate bone formation. Our work is aimed, in particular, at identifying a method to induce an increase in ERα gene expression in ERα-deficient cells. This should confirm that bone-forming osteoblasts, that are physiological targets for estrogen action, can also be a good target for a therapeutic approach aimed at restoring or increasing ERα expression. In the study presented here, we have shown the positive modulation of ERα mRNA expression in the TE85 human osteosarcoma cell line and in three of four human primary osteoblastic cells by the transfection of decoy molecules (DNA-102) against a sequence of distal promoter (−3258/−3157) of the ERα gene, previously described as a silencer in breast cancer cells (Penolazzi *et al.* 2000). After decoy treatment the strongest increase in ERα gene transcription was observed in the TE85 osteosarcoma cell line. In primary osteoblasts, where the investigation of upstream RNA levels was also performed, we demonstrated that expression of the ERα gene is mainly due to the activity of upstream promoters (P2/P3 and PF), in agreement with the observations of other authors (Grandien *et al.* 1995, Flouriot *et al.* 1998), and that, after decoy treatment, it increased in an appreciable manner. Even if the significance of the different ERα RNA isoforms and the cooperation of specific transcription

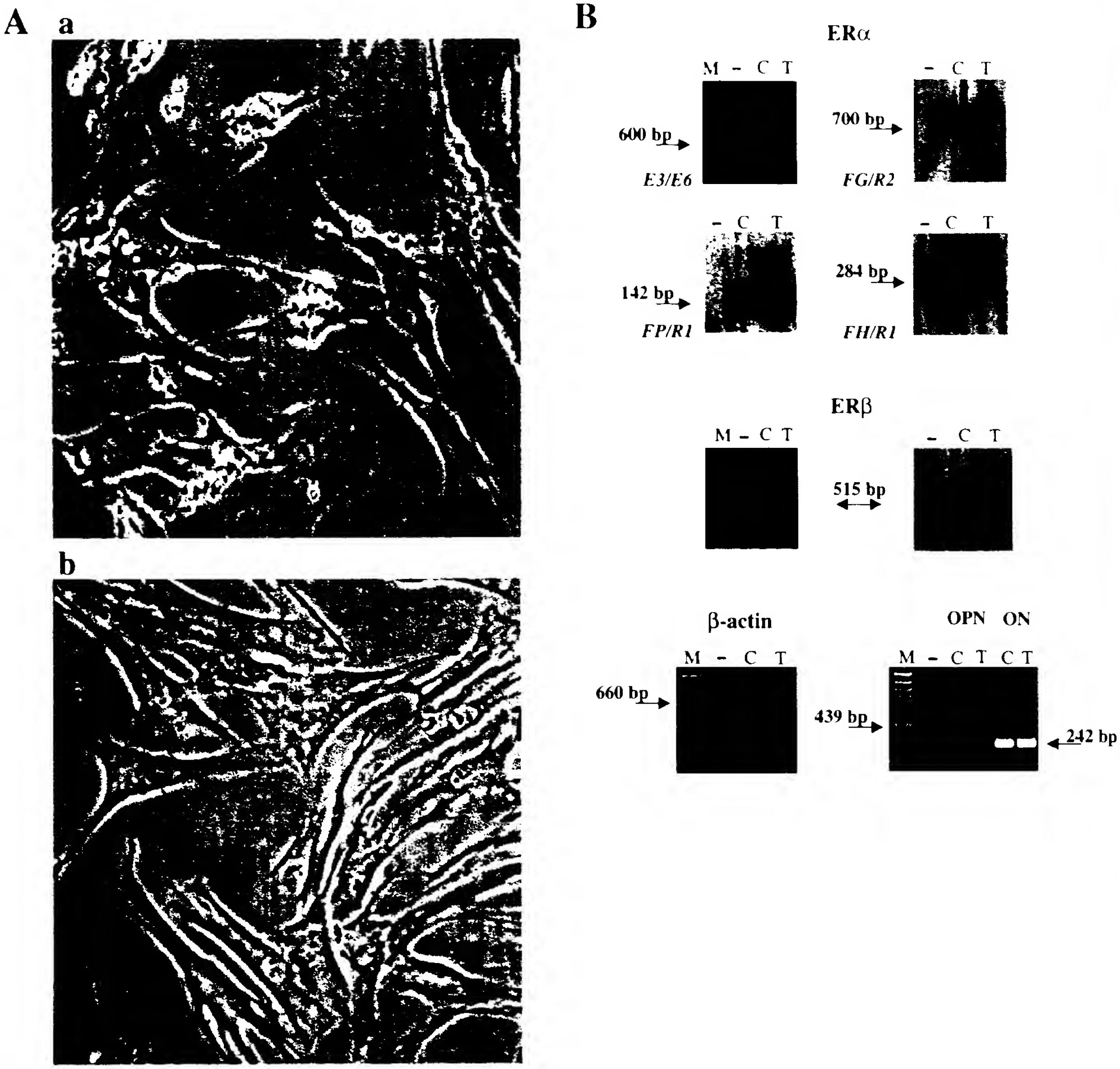


Figure 3 (A) Photomicrographs of primary osteoblasts treated with the DNA-102 decoy molecule complexed with cationic liposomes (a), or left untreated (b). Original magnification: × 100. (B) Effect of DNA-102 decoy on modulation of ERα, ERβ, OPN and osteonectin (ON) gene transcription. Osteoblastic cells isolated from osteopenic bone specimens obtained from four patients (3 women aged 65, 78 and 80 years, samples 1, 3 and 4 respectively; and one man aged 64, sample 2) were transfected with the DNA-102 decoy molecule (T) or were untreated (C). RT-PCR products of total ERα mRNA (E3/E6), ERα RNA 1 isoform (FG/R2), ERα RNA 2 and 3 isoforms (FP/R1) and ERα RNA H isoform (FH/R1) were separated in 1·8% agarose gel and stained with ethidium bromide. For the analysis of RNA isoforms, RT-PCR products were blotted onto nylon membrane and hybridized with the pGHER1 specific probe (Ponglikitmongkol *et al.* 1988). For ERβ mRNA, the specific probe used was pSG5-hERβ (Ogawa *et al.* 1998). A representative RT-PCR experiment corresponding to the expression of ERα, ERβ, OPN and ON mRNA from sample 1 is shown. All amplifications were compared with a negative control (primers without RNA), and the levels of ERα, OPN and ON mRNA were normalized against the β-actin mRNA content using a densitometric analysis. All RT-PCR products visible on agarose gel or the autoradiographic signals were then subjected to densitometry. The results are summarized in Tables 2 and 3. M, molecular weight marker.

factors for their expression awaits further investigation, it is likely that the complex promoter organization of ERα gene limits the cell species competent for the expression.

Our data suggest that the sequence extending from − 3258 to − 3157 may be considered critical for the lack of or decrease in ERα gene transcription in the bone

Table 2 The effect of DNA-102 decoy on estrogen receptor (ERα) gene transcription assessed by RT-PCR analysis. Total ERα mRNA results from the levels of type 1, type 2 and type H ERα mRNAs determined by semiquantitative RT-PCR as described in the experimental procedures. All samples (1–4) treated with DNA-102 (+) or untreated (−) were quantified in at least two independent experiments. The fold induction (Fold ind.) of gene transcription after decoy is also reported. The promoter utilization (after decoy treatment) is based on the expression levels of upstream ERα RNA isoforms (for P2 P3 and PF promoters) and canonical ERα transcript (for P1 promoter)

	Total ERα mRNA			Promoter usage		
	−	+	Fold ind.	P1	P2 P3	PF
Sample						
1	0·76	1·87	× 2·46	●	◍	◍
2	0·20	0·37	× 1·8	○	◍	●
3	0·37	0·46	× 1·2	○	◍	●
4	0·56	0·57	× 1·01		●	●

Solid circles, preferentially used; stippled circles, partially used; open circles, not used.

Table 3 The effect of DNA-102 decoy on osteopontin (OPN) and osteonectin (ON) gene transcription assessed by RT-PCR analysis. OPN and ON mRNA levels were determined by semiquantitative RT-PCR as described in the experimental procedures. All samples (1–4) treated with DNA-102 (+) or untreated (−) were quantified in at least two independent experiments. The fold induction (Fold ind.) of gene transcription is also reported

	OPN mRNA			ON mRNA		
	−	+	Fold ind	−	+	Fold ind.
Sample						
1	0·28	1·0	× 3·5	0·6	0·6	× 1·0
2	0·33	0·96	× 2·9	0·75	0·85	× 1·13
3	0·17	0·1	–	0·85	1·0	× 1·17
4	0·12	0·2	× 1·6	0·33	0·42	× 1·3

and that, when exogenously transfected, could compete with endogenous specific negative transcription factors. This is also in agreement with the data obtained in breast cancer cell lines (Penolazzi *et al.* 2000) and with the concept that the ERα gene promoters are under different controls.

It is interesting that the decoy-induced positive effect could also be observed on the expression of a bone differentiation marker, such as OPN, both in the TE85 cell line and in primary osteoblasts. By contrast, the cells analyzed remained ERβ negative after DNA-102 decoy treatment. Taken together, these results suggest that the approach here described may be considered an effective method to improve the osteoblastic phenotype through a mechanism in which ERβ does not appear to be involved.

The mechanism by which DNA-102 decoy brought about an increase in or activation of ERα gene expression is not clear. However, the fact that this change in expression was previously observed also in breast cancer cells after the same decoy treatment, suggests that DNA-102 decoy may act through modulation of DNA–protein binding or protein–protein interactions, stabilizing factors or conformation modifiers specific for ERα gene, and subtracting specific negative transcription factor(s) – nTF – that binds the sequence of DNA-102. Therefore, we speculate that DNA-102 decoy molecule, through reducing the nTF binding to its putative sequence inside DNA-102, would prevent an inhibitory signaling pathway on ERα gene transcription, favoring the positive control of ERα on transcription of target genes and inducing a more differentiated bone phenotype. Our experiments demonstrating that the promoter usage did not change when the cells were transfected with DNA-102 decoy molecule strengthen this hypothesis, suggesting that our decoy approach may intensify a committed osteoblastic phenotype.

Nevertheless, we cannot exclude the possibility that the DNA-102 decoy is able to induce osteoblastic differentiation independently of ERs and estrogen and that the increase in ERα and OPN expression may be a secondary event. Therefore, further investigations are required not only to quantitatively correlate the level of ERα gene transcription with ERα protein levels, but also to accurately analyze whether or not the ERα-mediated effect on osteoblastic differentiation, which is suggested here, is ligand dependent. Further investigation regarding the correlation between the increase in ERα gene expression and improvement in bone mass, in relation to specific clinical parameters, is also required to confirm the utility of the decoy approach here proposed. This may be of great significance for the development of new therapeutic strategies to improve bone mass in bone diseases such as postmenopausal osteoporosis which is characterized by a low bone mass and an increased risk of fracture (Rizzoli *et al.* 2001).

It is noteworthy that the study of regulatory mechanisms of ERα expression may contribute to a better understanding of the wide spectrum of effects of estrogen action in the bone microenvironment depending on the different ER isoforms (Rickard *et al.* 1999), the presence of the two orphan receptors that are closely related to the ERs, estrogen receptor-related receptors α and β (ERRα and ERRβ) (Vanacker *et al.* 1999), the balance between co-activators and co-repressors (Shibata *et al.* 1997), and the type of target DNAs (Rickard *et al.* 1999). In particular, because the interaction between ERα and ERβ is described (Bord *et al.* 2001, Compston 2001), it will be interesting to assess the DNA-102 decoy effect on osteoblast primary cultures that will express ERβ protein.

In addition, although estrogen appears to be the most important sex steroid involved in skeletal maturation and

mineralization (Rao & Murray 2000), osteoblast proliferation and differentiation are believed to be regulated by the combined effect of a key number of growth factors, cytokines and hormones that, alternatively, might mediate the effect of the DNA-102 decoy molecule.

In spite of the fact that there is limited information on the intermediate stages of the osteoblast differentiation pathway, by analyzing the effects of our decoy molecule on ERα, OPN and ON gene expression in primary cultures and identifing regulatory elements that maintain a specific bone phenotype via ER cell-specific gene expression, we can provide valuable information delineating the role of specific DNA–protein interactions on regulatory regions of the ERα gene in osteoblast differentiation.

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High Expression of the *Cap43* Gene in Infiltrating Macrophages of Human Renal Cell Carcinomas[1]

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ABSTRACT

We used suppression subtractive hybridization to identify highly expressed genes in the cancerous region of human renal cell carcinoma (RCC) compared with noncancerous tissue. Nine genes were identified to show increased expression in the cancerous region compared with the noncancerous region. The nine genes included *thymosin β4,* secreted protein acidic and rich in cysteine (*SPARC*), *Cap43, ceruloplasmin, serum amyloid A, osteopontin,* heat shock protein 90 (*HSP90*), *LOT1,* and *casein kinase I.* Of these 9 genes, *in situ* hybridization with 10 clinical samples consistently showed a strong expression of Cap43 mRNA in infiltrating macrophages in RCCs, but not in cancer cells proliferating in an alveolar pattern. However, Cap43 mRNA was also apparently detected in epithelial cells of the renal proximal tubuli in noncancerous tissue. The higher expression of the *Cap43* gene in the cancerous region of RCCs appears to depend on macrophage infiltration. Moreover, treatment with phorbol ester resulted in enhanced expression of the *Cap43* gene in human monocytic cells *in vitro*. The expression of the *Cap43* gene in infiltrating macrophages is discussed in association with the differentiated or activated status of monocyte/macrophage.

INTRODUCTION

RCC,[3] a common urogenital malignancy, is known as one of hypervascular and invasive tumors and often shows resistance to various chemotherapeutic treatments. Alteration of the *VHL* or *TSC* tumor suppressor genes and of the *MET* oncogene is thought to be associated with the formation of renal tumors (1–4). However, how the occurrence and progression of RCCs are induced at the molecular level remains unclear, for the most part. Moreover, both angiogenic and invasive phenotypes of RCC are thought to be associated with production of various angiogenic factors including vascular endothelial growth factor or basic fibroblast growth factor and altered expression of adhesion molecules and/or metalloproteinases (5–8). The isolation and determination of highly expressed genes in cancerous regions of RCCs are expected to be of particular importance. To understand the malignant phenotype of RCCs, various experimental methods have been established to compare patterns of gene expression, including differential display, serial analysis of gene expression, cDNA microarray, and SSH. In our present study, we isolated nine genes highly expressed in the cancerous region of human RCC tissue compared with the noncancerous region by SSH (9). Of the nine genes, we further examined the expression of one gene named *Cap43* in RCCs because *in situ* hybridization with RCC clinical samples consistently showed a strong expression of only Cap43 mRNA in infiltrating macrophages. The expression of the *Cap43* gene in human RCCs is discussed in association with malignant status of RCC and infiltrating macrophages.

MATERIALS AND METHODS

Samples. Noncancerous and cancerous tissue from the same case with a RCC were obtained from a 64-year-old Japanese male. Resected specimens from 10 cases (age range, 42–74 years) with RCCs who underwent operations in the Department of Urology at Kyusyu University Hospital between 1997 and 2000 were examined for dot blot analysis and Northern blot analysis. All surgical specimens were snap-frozen immediately after removal and stored at −80°C. Tumor tissue samples were obtained under an institutional review board-approved protocol, with subjects providing informed consent. Paraffin-embedded sections from 10 RCC cases including the above-mentioned 10 cases were obtained to perform *in situ* hybridization for the *Cap43* gene. Histological confirmation of the diagnosis was obtained in all cases.

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[3] The abbreviations used are: RCC, renal cell carcinoma; SSH, suppression subtractive hybridization; FBS, fetal bovine serum; PMA, phorbol 12-myristate 13-acetate; HSP, heat shock protein; SPARC, secreted protein acidic and rich in cysteine; TAM, tumor-associated macrophage; DIG, digoxigenin.

mRNA Isolation, cDNA Synthesis, and SSH. Total RNA from cancerous and noncancerous tissue was isolated by the guanidium isothiocyanate method. We purified mRNA using oligotex beads (Qiagen). cDNA was synthesized using SMART PCR cDNA synthesis (Clontech). SSH was performed between the noncancerous region (driver) and the cancerous tissue (tester) using the PCR select cDNA subtraction kit (Clontech) according to the manufacturer's recommendations.

Cloning and Sequencing of the Subtracted cDNA and Reverse Northern Dot Blot Analysis. The products from the secondary PCR (subtracted cDNA) were inserted into the PCRII-TOPO vector (TOPO TA cloning kit; Invitrogen), and ligated DNA was transfected into competent cells with selection for ampicillin resistance. One hundred clones were randomly selected and added to Luria-Bertani medium with ampicillin and grown at 37°C. Each plasmid DNA was prepared, and both strands of the isolated cDNA clones were sequenced using a Big-Dye Terminator Cycle Sequencing kit (Applied Biosystems) and a DNA sequencing system (model 377; Applied Biosystems) with M13 universal primers. For sequence analysis, the BLAST program at the National Center for Biotechnology Information was used. We designed specific primers for 32 genes that were identified independently, and a sufficient amount of their PCR products was spotted onto a nylon Hybond-N membrane (Amersham) and hybridized to ^{32}P-labeled cDNA probes from both noncancerous and cancerous tissue. Radioactivity was detected using a Fujix Bas 2000 bio-image analyzer, and the expression level of each gene was normalized to β-actin expression.

Cell Culture. Human monocytic U937 cells were cultured in RPMI 1640 supplemented with 10% FBS. These cells were incubated in culture medium supplemented with 1% FBS for 16 h and treated with or without 10 μg/ml PMA, 100 ng/ml lipopolysaccharide, 1000 IU/ml IFN-α, and 50 ng/ml IFN-γ for 6 h.

RNA Dot Blot and Northern Blot Analyses. For dot blot analysis, 1 μg of total RNA obtained from both noncancerous and cancerous tissue of 10 RCC cases was spotted onto a nylon membrane (Hybond N+; Amersham), respectively. For Northern blot analysis, total RNA (10 μg) was electrophoresed on a 1% agarose gel containing 2.2 μM formaldehyde and transferred onto a nylon membrane. Each membrane was cross-linked with UV light at 0.25 J/cm^2 with FLUO-LINK (Viler Lourmat, Marne-La-Vallee, France). The probes used were fragments amplified by PCR using specific primers. The primers for each gene were as follows: (*a*) *Cap43,* coding strand primer 5′-GCTACAACCCCCTCTTCAAC-3′ and non-coding strand primer 5′-GGGTTCACGTTGATAAGGAC-3′; (*b*) *thymosin β4,* coding strand primer 5′-GCTTCGCTTTTCCTCCGCTA-3′ and non-coding strand primer 5′-CGCACGCCTCATTACGATTC-3′; (*c*) *SPARC,* coding strand primer 5′-TCTTTGCCACAAAGTGCACC-3′ and non-coding strand primer 5′-CCAGTGTACAGGGAAGATG-3′; (*d*) *osteopontin,* coding strand primer 5′-CAGTCTGATGAGTCTCACCA-3′ and non-coding strand primer 5′-ATGCACCATTCAACTCCTCG-3′; and (*e*) β-*actin,* coding strand primer 5′-TCCTGTGGCATCCACGAAAC-3′ and non-coding strand primer 5′-GAAGCATTTGCGGTGGACGA-3′. For RNA dot blot and Northern blot analysis, the procedure after hybridization was performed as described previously (10).

***In Situ* Hybridization.** Fragments amplified by PCR using specific primers of *Cap43* were cloned into pCR2.1 (Invitrogen) in two orientations, and DIG-labeled sense and antisense RNA probes were synthesized with T7 RNA polymerase using a DIG-RNA Labeling Kit (Boehringer Mannheim) according to the manufacturer's instructions. The procedure for *in situ* hybridization was as described previously (10).

Immunohistochemistry. Resected specimens of RCCs were fixed in 10% formalin solution, processed routinely, and embedded in paraffin. Six-μm-thick sections were stained immunohistochemically using an avidin-biotinylated peroxidase complex method with a mouse monoclonal antibody against the macrophage marker CD68 (Dako Glostrup, Denmark). The sections were counterstained lightly with hematoxylin (10).

Quantification of Macrophages. In each case, macrophage infiltration was assessed microscopically in the three hottest after a brief scan of the entire section at low power, and the number of macrophages/microscopic field (×400 magnification) was recorded (10).

RESULTS

Differential Expression of Identified cDNA between the Cancerous and Noncancerous Regions in Human RCC. By SSH, we isolated clones that were relatively highly expressed in the cancerous region compared with the noncancerous region from a surgically resected sample of one case with RCC (see "Materials and Methods"). One hundred clones inserted into the PCRII-TOPO vector were randomly selected, and then they were sequenced. Thirty-two genes identified independently were further tested by reverse Northern dot blot hybridization. Of the 32 genes, the expression of 9 genes was higher in the cancerous region than in the noncancerous region of this case (Fig. 1). These nine genes included *LOT1, HSP90, casein kinase I, thymosin β4, serum amyloid A, ceruloplasmin, SPARC, Cap43,* and *osteopontin*. The expression level of each gene was normalized to β-actin expression. The expression of these nine genes was 1.4–4.3-fold higher in the cancerous region than in the noncancerous region (Table 1).

Specificity of Highly Expressed Genes in RCCs. To examine whether the nine genes are also highly expressed in the cancerous region compared with the noncancerous region in other cases with RCCs, RNA dot blot analysis was performed with 10 RCC cases. Fig. 2*A* shows an example of experimental data for five genes. Of 10 cases, a higher expression of thymosin β4 (8 cases), SPARC (7 cases), Cap43 (6 cases), and ceruloplasmin (6 cases) was seen in the cancerous region as compared with the noncancerous region. The expression levels of Cap43, thymosin β4, SPARC, ceruloplasmin, serum amyloid A, HSP90, and osteopontin mRNA in the cancerous and noncancerous regions were normalized to β-actin levels in 10 cases (Table 2). The expression levels of serum amyloid A, osteopontin, and HSP90 mRNA appeared to be similar between the cancerous and noncancerous regions in 5–6 of 10 cases with RCCs (Table 2). The expression of LOT1 mRNA was not observed by dot blot analysis, and the expression of casein kinase I appeared at similar levels in the cancerous and noncan-

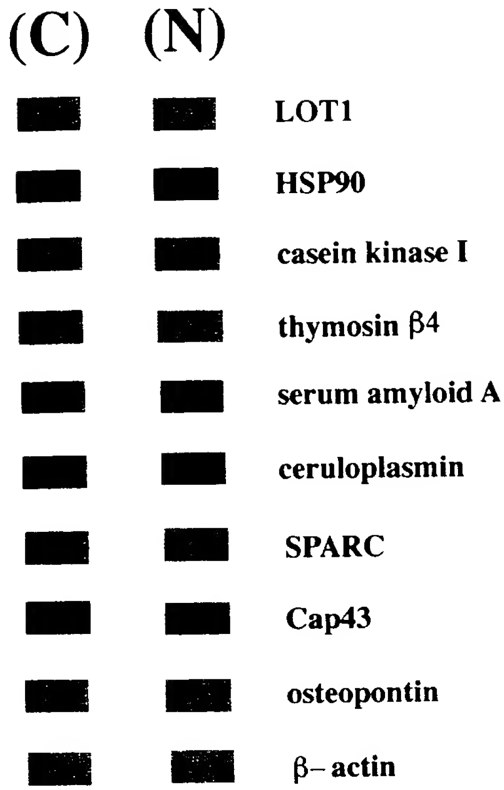

Fig. 1 Reverse Northern dot blot analysis for one case with RCC in which SSH was performed. Of 32 genes identified by SSH and sequence analysis, 9 genes showed altered expression between the noncancerous (*N*) and cancerous region (*C*) in RCCs. Specific primers of those 32 genes were designed, and a sufficient amount of those PCR products was double-spotted onto a nylon Hybond-N membrane and hybridized to ^{32}P-labeled cDNA probes synthesized using gene-specific primers from both the noncancerous and the cancerous tissue. β-Actin expression was the control. Radioactivity was detected and normalized to β-actin expression (see also Table 1).

Table 1 Genes that were highly expressed in the cancerous region of RCC using a BLAST search of the public databases

Genes	C:N ratio[a]	Appearance[b]	Accession no.[c]
LOT1	3.0	1	U72621
HSP90	1.9	1	X15183
Casein kinase I γ 3L	3.1	1	AF049090
Thymosin β4	2.1	1	E01650
Serum amyloid A	2.4	1	NM 000331
Ceruloplasmin	1.4	1	NM 000096
SPARC	4.3	1	NM 003118
Cap43	2.0	2	AF004162
Osteopontin	2.4	1	J04765

[a] The increase in the signal obtained with the cDNA probe from the cancerous tissue (C) compared with the cDNA probe from the noncancerous tissue (N) by reverse Northern dot blot analysis (see Fig. 1).

[b] The number of times that the same gene appears in the analysis of 100 clones.

[c] The accession number in the public databases.

cerous regions in all 10 cases (data not shown). We further examined mRNA levels of Cap43, thymosin β4, and SPARC in four cases by Northern blot analysis (Fig. 2*B*). Both dot blot and Northern blot analyses consistently showed an increased expression of Cap43 in the cancerous region compared with the noncancerous region in cases 7 and 8 (Fig. 2, *A* and *B*). By contrast, the expression of Cap43 was higher in the noncancerous region than in the cancerous region in cases 6 and 9. Similar results were obtained when the expressions of thymosin β4 and SPARC were compared between dot blot and Northern blot analysis (Fig. 2, *A* and *B*).

Detection of Cap43 mRNA in Infiltrating Macrophages of RCC Tissues. We first examined whether these nine genes were expressed in cancer cells themselves or in other stroma cells in RCCs. The localization of mRNA of these nine genes in RCC tissue was assessed by *in situ* hybridization with each antisense RNA probe in two cases. Of the nine genes, we observed a positive expression of only the *Cap43* gene in infiltrating macrophage-like cells in the cancerous regions (data not shown). Therefore, we further examined the expression of Cap43 mRNA in eight other RCC cases.

The expression of the *Cap43* gene was determined in both noncancerous regions and cancerous regions by *in situ* hybridization with an antisense Cap43 RNA probe in 10 RCC cases. Fig. 3 shows one example of *in situ* hybridization. Expression of the *Cap43* gene was detected mainly in epithelial cells of the proximal tubuli (Fig. 3*A*) in the noncancerous regions of all 10 cases. In contrast, a positive staining for the *Cap43* gene was observed only in large and round cells that have a rich cytoplasm, especially near the capsule in the cancerous region of 6 of 10 cases (Fig. 3*C*), and not within cancer cells proliferating in an alveolar pattern in the cancerous regions (Fig. 3*B*). Positive staining for the *Cap43* gene was also found in large, round cells scattered within the mass of cancer cells (data not shown).

In the cancerous regions of RCCs and other human tumors, macrophages are often observed in the stroma (11). The appearance of macrophages was determined by immunostaining with anti-CD68 antibody, which is an antibody specific for macrophages. No apparent infiltration of macrophages is observed in the noncancerous region of RCCs, whereas many CD68-positive cells are observed, mainly near the capsule in the cancerous regions. When examined using a series of specimens, most cells stained for Cap43 mRNA were also positive for anti-CD68 antibody (Fig. 3*D*). We also examined immunostaining with anti-CD3 antibody, which is an antibody for T lymphocytes. However, the distribution of cells stained for Cap43 mRNA was different from that of CD3-positive cells.[4] Cells that stained positively for Cap43 mRNA in the stroma could be predominantly macrophages infiltrating the cancerous regions.

Cap43 Expression and Macrophage Infiltration in the Cancerous Region of RCC Cases. We next examined whether expression of the *Cap43* gene in the cancerous region was correlated with macrophage infiltration. As shown in Fig. 2, dot blot analysis in 10 cases demonstrated Cap43 mRNA expression at various levels. The correlation between Cap43 mRNA levels in the cancerous region and average number of macrophages infiltrating the cancerous region was assessed. We

[4] A. Nishie and M. Kuwano, unpublished data.

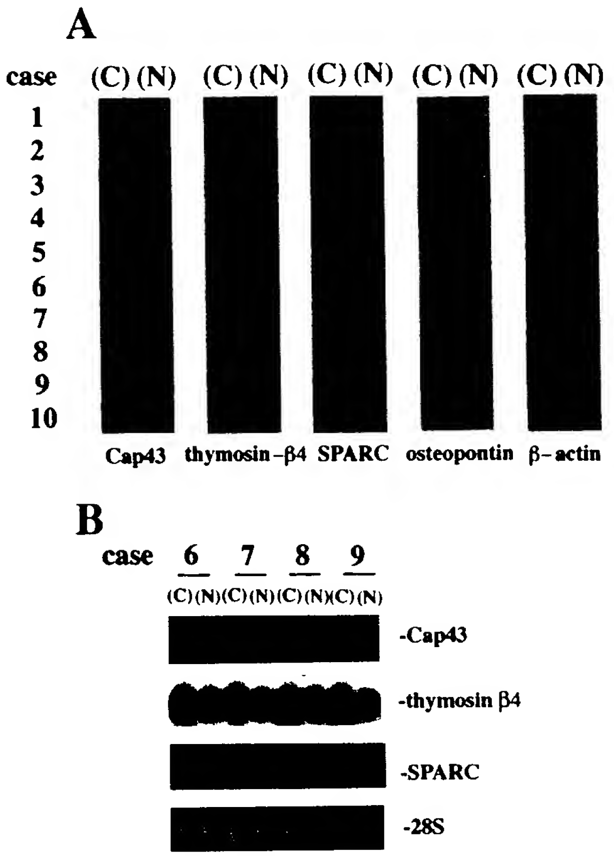

Fig. 2 *A*, RNA dot blot analysis of *Cap43, thymosin* β*4, SPARC*, and *osteopontin* genes in 10 RCC cases. One μg of total RNA obtained from both noncancerous (*N*) and cancerous tissue (*C*) of 10 RCC cases was spotted onto a nylon membrane. The membrane was hybridized to ^{32}P-labeled double-stranded DNA probes produced by the Klenow fragment, and radioactivity was detected and normalized to β-actin expression. *B*, Northern blot analysis of four RCC cases including cases 6–9. Ten μg of total RNA obtained from both noncancerous (*N*) and cancerous (*C*) tissue of four RCC cases were transferred onto a nylon membrane. The membrane was hybridized to ^{32}P-labeled double-stranded DNA probes produced by the Klenow fragment. The radioactivity was normalized to each rRNA.

Table 2 Expression patterns of identified genes between the cancerous (C) and their noncancerous regions (N) in 10 RCC cases

Genes	C > N[a]	C = N[b]	C < N[c]
Thymosin β*4*	8	0	2
SPARC	7	2	1
Ceruroplasmin	6	3	1
Cap43	6	2	2
Serum amyloid A	3	5	2
Osteopontin	2	6	2
HSP90	1	5	4

[a] The number of cases in which each gene is highly expressed in the cancerous region compared with the noncancerous region.

[b] The number of cases in which each gene is equally expressed between the cancerous region and the noncancerous region.

[c] The number of cases in which each gene is highly expressed in the noncancerous region compared with the cancerous region.

observed a significant correlation ($r = 0.640$) between Cap43 mRNA levels and macrophage infiltration (Fig. 4).

Up-Regulation of the *Cap43* Gene in Human Monocytic Cells by a Differentiating Agent. *In situ* hybridization analysis demonstrated an apparent expression of the *Cap43* gene in macrophages in the cancerous region of six RCC cases examined. We further examined whether the expression of the *Cap43* gene was induced in human monocytic cells by various activating agents. The expression of Cap43 mRNA was specifically increased three times by PMA (Fig. 5), consistent with the findings of a previous study (12). No apparent change in Cap43 mRNA levels was observed using lipopolysaccharide, IFN-α, and IFN-γ.

DISCUSSION

In this study, we showed a number of differentially expressed genes in human RCC and described the usefulness of SSH with material obtained from primary cancer tissue. Reverse Northern dot blot analysis was performed to reduce the number of false positive clones. Nine of 32 genes identified by sequencing were highly expressed in the cancerous region compared with the noncancerous region in RCC. Sequencing analysis of the nine clones showed a high degree of homology with already known genes. Of the nine genes, the expressions of *SPARC, thymosin* β*4*, and *ceruloplasmin* were higher in the cancerous region than in the noncancerous region in more than 6 of 10 cases when surgically resected specimens were analyzed. *SPARC, thymosin* β*4*, and *ceruloplasmin* genes were reported to be closely associated with malignancy or angiogenesis in various kinds of tumors (13–22).

The *Cap43* gene was isolated as a gene induced with nickel compounds in human bronchoalveolar epithelial cells using the differential display technique (23). This gene expresses a 3.0-kb mRNA encoding a 43-kDa protein and has three human homologues (RTP, Drg-1, and rit42; Ref. 23). The Cap43 protein contains a new motif consisting of 10 amino acids repeated three times in the COOH terminus (23). The expression of the *Cap43* gene is relatively low in the cancerous region compared with the noncancerous region in colon, breast, and prostate cancers (24, 25). Two studies have shown that Cap43 might be involved in growth, differentiation, and metastasis of tumor cells (25, 26). Contrary to these previous studies, the expression of Cap43 mRNA was higher in the cancerous region than in the noncancerous region in 6 of 10 cases with RCCs. *In situ* hybridization showed that a strong staining for the *Cap43* gene was observed mainly in the stroma cells, especially near the capsule within the cancerous region, and that most of the Cap43-positive stromal cells were macrophages infiltrating the RCCs. Moreover, we observed a significant correlation between Cap43 mRNA levels and macrophage infiltration in the cancerous region of RCCs (Fig. 4). Expression levels of the *Cap43* gene in the cancerous regions of RCCs thus appear to be due to the number of Cap43-positive macrophages.

Infiltration of TAMs is often observed in RCCs (11). Macrophage infiltration is closely associated with angiogenesis and malignancy in various human tumor types, such as breast cancer, colon cancer, brain tumor, melanoma, and non-Hodgkin's lymphoma (10, 27–30). Furthermore, TAMs infiltrating human breast cancers (27) and malignant melanomas

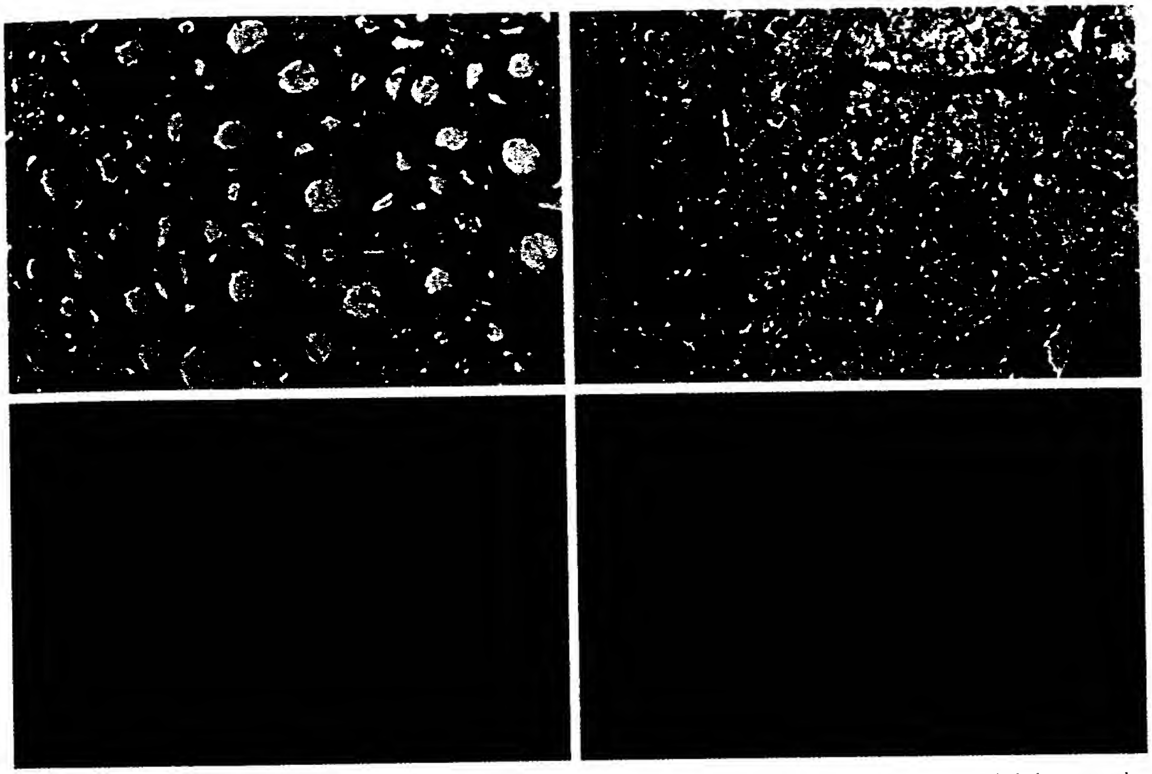

Fig. 3 Detection of Cap43 mRNA by *in situ* hybridization and immunohistochemical staining for CD68 in RCCs. DIG-labeled sense and antisense RNA probes of Cap43 were synthesized and hybridized to the RCC section of case 7. *A* shows the noncancerous tissue using an antisense RNA probe. *B* and *C* show the cancerous tissue using an antisense RNA probe. Cap43 mRNA was partially detected in the epithelial cells of the proximal tubuli (*A*). A strong staining for the *Cap43* gene was observed particularly in large, round cells with a rich cytoplasm, especially near the capsule (*C*), and not within cancer cells proliferating in an alveolar pattern (*B*). No significant signal was observed using a sense RNA probe. When the sections were treated with RNase in the process of washing, the result was the same, alhough the entire signal was reduced. Sections were stained with anti-CD68 antibody using an avidin-biotinylated peroxidase complex method. *D* illustrates the result of case 7. No apparent macrophage infiltration was observed in the noncancerous region, whereas prominent infiltration was observed in the cancerous region, especially near the capsule. The distribution of macrophages was almost consistent with that of cells stained for Cap43 mRNA. Magnification: ×200, *A* and *B*; ×100, *C* and *D*.

(31) showed high expression of thymidine phosphorylase, and those cells infiltrating human gliomas showed high expression of heme oxygenase-1 (10). These studies suggested that the appearance of thymidine phosphorylase-positive or heme oxygenase-1-positive macrophages is somehow associated with malignant and/or angiogenic status in the above human tumors. Piquemal *et al.* (12) has shown that differentiation of human monocytic cells by phorbol ester results in increased expression of the *Cap43* gene. We also confirmed that monocytic cells enhanced expression of the *Cap43* gene in response to PMA (Fig. 4). Differentiation of monocytic cells thus appeared to accompany enhanced expression of the *Cap43* gene. The role of the *Cap43* gene in macrophages remains unclear, but this gene is expected to be a marker of mature form during differentiation of the mononuclear phagocyte system and also of TAMs. From our present study, the appearance of Cap43-positive macrophages might have an important role in the malignant or angiogenic status of RCCs. Further study is needed to investigate the biological role of the *Cap43* gene in macrophages.

The expression of the *Cap43* gene was not observed in renal cancer cells themselves, whereas it was positively detected in epithelial cells of the proximal tubuli in the noncancerous region of all 10 cases (Fig. 3, *A* and *B*). Considering the fact that

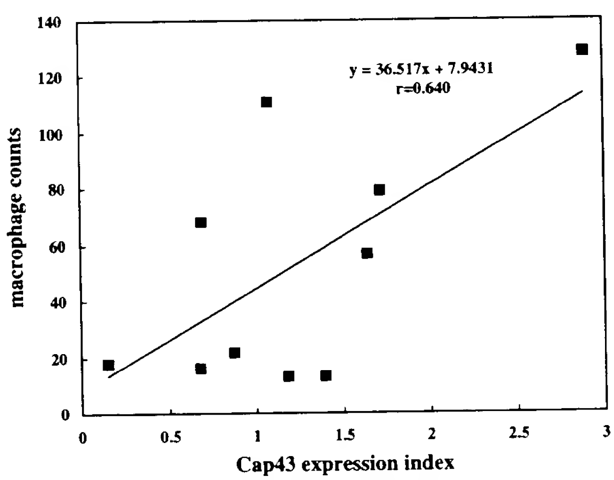

Fig. 4 Correlation between expression of Cap43 mRNA and macrophage infiltration. Cap43 mRNA levels in the cancerous region are based on the data in Fig. 2. Macrophage counts in the cancerous region were assessed microscopically in the three, and the average number of macrophages/microscopic field (×400 magnification) was recorded. Expression of Cap43 mRNA was closely correlated with macrophage infiltration in 10 RCC cases.

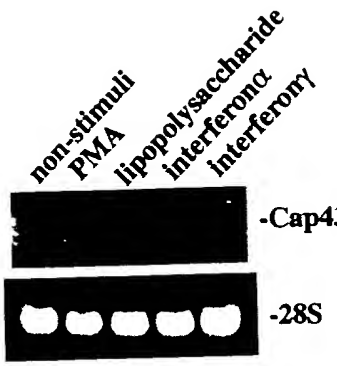

Fig. 5 Induction of Cap43 mRNA by stimulation with PMA. Human myelomonocytic cells were incubated in the culture medium with 1% FBS for 16 h and treated with or without 10 μg/ml PMA, 100 ng/ml lipopolysaccharide, 1000 IU/ml IFN-α, and 50 ng/ml IFN-γ for 6 h. Ten μg of total RNA obtained from treated cells were isolated and subjected to Northern blot analysis. The expression of Cap43 mRNA was increased 3-fold by stimulation with PMA.

RCCs originate from epithelial cells of the proximal tubuli, expression of the *Cap43* gene might disappear when malignant phenotypes are acquired in human RCCs. This study has also shown that Cap43 might be involved in growth and cell differentiation in RCCs as well as colon, breast, and prostate cancers (25, 26).

In conclusion, we isolated nine genes that were highly expressed in the cancerous region of RCC compared with noncancerous tissue. Of these nine genes, one gene named *Cap43* appeared to be somehow associated with the malignant characteristics of cancer cells because the *Cap43* gene was expressed in normal epithelial cells of the proximal tubuli but not in renal cancer cells. Moreover, Cap43 mRNA was highly expressed in infiltrating macrophages in the cancerous region of RCCs, suggesting that Cap43 is a novel biochemical marker for differentiation of renal cells or monocytes/macrophages. The expression of the *Cap43* gene in the cancerous region of RCCs could reflect macrophage infiltration. Further study is required to understand how the expression of the *Cap43* gene in infiltrating macrophages could be associated with the acquisition of malignant phenotypes of RCC.

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malignant behavior both *in vitro* and *in vivo*. *In vitro*, the cell growth curve analysis indicated that the cells transfected with NCAM had a slow growth rate than the parental cells. The cell cycle analysis with flow cytometer showed that the NCAM transfected cells had a less population in S phase than its counterparts (32.5% v.s. 40.5%). In addition, the rate of PCNA positive staining in NCAM positive cells was lower than control (63.4% v.s. 98.1%). Furthermore, the invasiveness of cells was analyzed with Boyden chamber assay. The results showed that the numbers of NCAM positive cells penetrated through a matrigel-coated filter were less than that of the parental cells (mean cell number 103 v.s. 223), which could be blocked by monoclonal anti-NCAM. When intracerebrally inoculated the cells, while the tumor incidence of parental cells was 76.9% with a large size of tumors, the NCAM transfectants was 31.3% with small tumors. Taken together, the NCAM could inhibit the growth and invasion of malignant glioma cells.

#3964 Prostate-specific gene expression and androgen regulation of lentiviral vectors containing elements of the probasin promoter. Duan Yu, Paul S. Rennie, Martin E. Gleave, Colleen C. Nelson, and William W. Jia. *The Prostate Center at Vancouver General Hospital and the Department of Surgery, University of British Columbia, Vancouver, BC, Canada.*

The concept of using a tissue-specific promoter to express exogenous therapeutic genes in prostate cancers offers an attractive way of potentially targeting locally advanced and metastatic tumors for eradication or control. In addition to a prostate-specific promoter, targeted gene therapy for prostate cancer requires the development of a suitable gene delivery system. In the present study, we investigated the prostate-specific expression and androgen regulation of three probasin promoter (PB) constructs (kindly provided by Dr. R.J. Matusik) linked to the enhanced green fluorescent protein reporter (EGFP) and inserted into a human immunodeficiency virus (HIV-1) derived lentiviral (Lt) vector, which is able to infect efficiently and to express exogenous transcripts in both divided and non-divided cells. The lentiviral-PB promoter/reporter vectors Lt-(-426/+28)PB-EFGP, Lt-(-286/+28)PB-EGFP, and Lt-ARR_2PB (essentially a dimer of -286/+28)-EGFP, together with a control lentiviral-CMV promoter/reporter (Lt-CMV-EGFP), were constructed and tested for relative EGFP expression after infection *in vitro* of human prostate cancer cell lines (LNCaP, PC-3, and Du145 cells) and non-prostate lines (Cos-1, HeLa, and MCF-7 cells). Although high EGFP expression was seen at near equivalent levels in both prostate and non-prostate cells infected with control Lt-CMV-EGFP, with the PB lentiviral vectors EGFP expression was only observed in LNCaP cells with a small amount seen in PC-3 cells. The expression level of EFGP in cells infected with Lt-ARR_2PB-EGFP was the highest among the three PB promoter constructs tested. In LNCaP cells growing in charcoal-stripped media, a maximal 5-fold increase in EGFP expression was seen between 16-24 hours after treatment with androgen (10 nM DHT). The intensity of EGFP expression in these cells declined by 50% after 4 days of androgen withdrawal. Approximately 3-times more LNCaP cells expressed EGFP than did PC-3 cells and the mean intensity in LNCaP cells was about 6-fold greater. Transfection of androgen receptor expression vectors and treatment with androgens (10nM DHT) raised the intensity of EGFP expression in LNCaP and PC-3 cells but did not alter expression levels in the other non-prostate cell lines tested. Our results indicate that lentiviral vectors containing elements of the PB promoter can express an exogenous reporter gene in a prostate-specific and androgen-regulated manner and therefore may be excellent tools for targeted gene therapy of prostate cancer. (Supported by grants from the Terry Fox Foundation and NCI of Canada).

#3965 Gene transfer studies using the synthetic polyamide Syn3 to enhance intravesical adenoviral gene transfer in the urothelium and superficial bladder tumor independent of CAR expression. Charles J. Rosser, Robert J. Connor, Jain-Hua Zhou, Bogdan Czerniak, Xinqiao Zhang, Heidrun Engler, Motoyoshi Tanaka, Li Dong, Colin P. N. Dinney, H. Barton Grossman, Douglas Cromeens, Clifton Stephens, Daniel Maneval, and William F. Benedict. *UT MD Anderson Cancer Center, Houston, TX, and Canji, Inc., San Diego, CA.*

Adenovirus-mediated gene therapy of bladder diseases has been limited by the inability to efficiently transduce the urothelium. We have demonstrated that the addition of the polyamide,Syn3, to the adenoviral formulation can potently enhance adenoviral transgene expression in the urothelium (Connor et al., *Gene Therapy* 8: 41-48, 2001). We have expanded these initial studies to optimize gene transfer and expression using an orthotopic tumor model, as well as to study Syn3-rAd transgene expression in the urothelium of large animals. We have shown in rodents that 1h administration of rAd-β-gal in Syn3 (1 mg/ml) on 2 consecutive days provides more complete gene transfer and higher levels of expression compared to a single administration of rAd-β-gal-Syn3 at 1 mg/ml. We have also examined the ability of Syn3 to enhance adenoviral gene expression in a non-rodent species. Administration of an rAd-β-gal (1.2×10^{11} PN/ml) in a Syn3 formulation (1 mg/ml) yielded high levels of adenoviral transgene expression in the urothelium of the Yorkshire pig. Thus, it is anticipated that Syn3 will enhance adenoviral transgene expression in a variety of species, including humans. We also tested Syn3 in an orthotopic tumor model we recently developed (Watanabe et al., *Human Gene Therapy* 7: 1575-80, 2000). Using this mouse model, we demonstrate that two consecutive 1h administrations of rAd-β-gal in a Syn3 formulation resulted in complete transduction of the superficial tumor and the entire urothelium. We have also determined that Syn3 can potently increase gene transfer and expression to superficial bladder tumors (T24) that express little or no CAR (Coxsackie-Adenovirus Receptor). Since low CAR expression on superficial bladder tumors has been postulated to limit the success of adenoviral gene therapy, Syn3 may alleviate this barrier to successful gene transfer. To date little or no cytotoxicity has been observed in the bladders or other organs examined from mice, rats, rabbits, dogs and pigs. Blood chemistries from treated animals were also in the normal range following Syn3-rAd treatment. Therefore, it is anticipated that Syn3 will be a viable formulation enhancing agent to potentiate the use of adenoviral vectors for the treatment of superficial bladder cancer.

#3966 Delivery of bioactive ribozymes by an RNA-stabilizing polyethylenimine (PEI) efficiently down-regulates gene expression *in vitro* and *in vivo*. Achim Michael Aigner, Dagmar Fischer, Thomas Merdan, Carola Brus, Thomas Kissel, and Frank Czubayko. *Department of Pharmacology, Philipps-University, Marburg, Germany, and Department of Pharmaceutics and Biopharmacy, Philipps-University, Marburg, Germany.*

The sequence-specific cleavage of RNA molecules through ribozyme-targeting is particularly attractive since it allows the effective abrogation of the expression of any selected protein. Therefore, ribozymes represent potentially interesting therapeutic agents in tumor therapy and offer powerful strategies in proteomics / target validation applications to analyse the functions of potential genes of interest, e.g. in tumor biology. So far, however, use of enzymatically active RNA molecules like hammerhead ribozymes has without chemical modification been severely hampered by ribozyme instability and poor cellular uptake. Here, we present a method for protection and cellular delivery of bioactive ribozymes by complexation with a low molecular weight polyethylenimine (PEI). Polyethylenimines are synthetic branched polymers with high cationic charge density which form non-covalent complexes with nucleic acids and have been used as DNA transfection reagents. We show that low molecular weight polyethylenimines allow complete stabilization of ribozymes or any RNA against degradation. Upon their highly efficient cellular uptake, non-toxic PEI-complexed ribozymes display intracellular bioactivity already at low concentrations as demonstrated by down-regulation of two different genes in different cell lines. In particular, we describe a ~ 60 % depletion of an FGF-binding protein (FGF-BP) which has been described previously as rate-limiting for tumor growth and metastasis in squamous cell carcinomas and serves as an "angiogenic switch molecule". Likewise, the growth factor pleiotrophin (PTN) which is overexpressed in several tumors and which can be rate-limiting for tumor growth and angiogenesis, is efficiently downregulated in tissue culture upon targeting with PEI-complexed PTN ribozymes. Most strikingly, *in vivo* delivery of PEI-complexed ribozymes against PTN results in marked reduction of tumor growth and of intratumoral PTN levels in a mouse xenograft model. Thus, in this study we describe a novel, widely applicable method for exogenous delivery of any bioactive RNA ribozyme *in vitro* and *in vivo* without chemical modification.

#3967 Visualization of prostate cancer metastasis in living mice by a targeted gene transfer vector and optical imaging. Lily Wu, Jason Yeates Adams, Makoto Sato, Mai Johnson, Frank Berger, Sam Gambhir, Michael Carey, and Luisa Iruela-Arispe. *UCLA School of Medicine, Los Angeles, CA.*

Gene therapy represents a potential alternative or adjuvant to existing strategies in the treatment of advanced, androgen-independent (AI) prostate cancer. Non-invasive imaging modalities and transcriptional targeting have emerged as two tools to facilitate the rational design and improve upon the safety of vector-based cancer gene therapy. Here we demonstrate, for the first time, the ability to identify metastases in a human prostate cancer model, employing a prostate-specific adenovirus vector (AdPSE-BC-luc) and a charge coupled device (CCD) optical imaging system. We monitored the location, magnitude, and kinetics of luciferase expression in xenograft models of both androgen-dependent (AD) and androgen-independent (AI) prostate cancer. AdPSE-BC-luc, which expresses luciferase from an enhanced prostate specific PSA promoter, restricted expression in the liver and displayed 200,000-fold lower activity compared to constitutive AdCMV-luc after systemic tail-vein injection. On the other hand, intra-tumoral administrated AdPSE-BC-luc mediated relative robust signal in LAPC-4 and LAPC-9 human prostate cancer xenografts. Moreover, the expression is 9-fold higher in the advanced stage of AI than in AD LAPC-4 tumors. The elevated luciferase expression pattern paralleled the respective endogenous PSA expression pattern in LAPC-4 tumors. Repetitive imaging over a 3-week period after intra-tumoral injection of 1.8x10exp9 AdPSE-BC-luc revealed that the virus could locate and illuminate metastases in the lung and spine. Follow-up analysis of these lesions by histology and immunohistochemisty confirmed they were human neoplastic lesions. These results demonstrate the targeting ability of the prostate-specific vector and the utility of a non-invasive imaging modality in therapeutic and diagnostic strategies to manage prostate cancer.

#3968 A novel tissue-specific and tumor-restrictive human osteonectin promoter-mediated gene therapy for treatment of androgen-independent human prostate cancer. Chia-Ling Hsieh, Haiyen E. Zhau, and Leland W. K. Chung. *Department of Urology, Molecular Urology and Therapeutic Program, Emory University School of Medicine, Atlanta, GA.*

Stromal-epithelial interaction is important for the control of local prostate tumor growth and its distant metastasis. One approach to prostate cancer metastasis is the development of an effective gene therapy strategy for the delivery and expression of therapeutic genes specifically at the tumor site, with expression

both in tumor epithelium and adjacent stroma. We evaluated a number of promoters that regulate non-collagenous bone matrix proteins for their potential to drive the expression of therapeutic genes in tumors without affecting adjacent normal tissues. In this communication, we demonstrated that human osteonectin (hON) mRNA, detected by RT-PCR, was expressed in prostate cancer cell lines and tumor specimens. hON protein was also found to be expressed by human prostate tumor epithelium and its surrounding prostate and bone stromal cells as evaluated by immunohistochemistry. To achieve effective transgene delivery co-targeting tumor and stroma, we generated a highly active 580 bp hON promoter (522E) containing only positive transcriptional regulatory elements defined from both the promoter (bp 166 to 130 and bp 120 to 50) and exon 1 (bp +40 to +62) region of the hON gene. DNaseI footprint analysis demonstrated abundant transcriptional factors in the osteoblastic cell line MG63 and in androgen-independent and metastatic human prostate cancer cell lines with the propensity to metastasize to the skeleton, such as PC3M, but not in androgen-dependent LNCaP cells specifically bound on this exonic enhancer. When cells were infected with Ad-522E-TK, TK was expressed at high levels in androgen-independent and metastatic human prostate cancer cell lines of the DU145 and PC3 lineages, but lower hON activity was detected in the androgen-dependent LNCaP cells. In addition, Ad-522E-TK was also found to abolish the growth of PC3M and MG-63 *in vitro* and eliminate the growth of established PC3M tumors *in vivo* upon the addition of GCV. Because of the heterogeneity of human prostate tumors, the hON promoter (522E) may be a useful adjunct to other tissue-specific and tumor-restrictive promoters to drive therapeutic genes for the treatment of localized and metastatic prostate cancers.

#3969 Pro-apoptotic and anti-tumor activities of adenovirus-mediated p202 gene transfer. Duen-Hwa Yan, Yi Ding, Yong Wen, Bill Spohn, Li Wang, Weiya Xia, Ka Yin Kwong, Ruping Shao, Zheng Li, Gabriel Hortobagyi, and Mien-Chie Hung. *M. D. Anderson Cancer Center, Houston, TX.*

p202, an interferon-inducible protein, is a member of the murine 200-amino acid repeat family. Ectopic expression of p202 in human breast cancer cells resulted in growth inhibition and sensitization to TNF-α-induced apoptosis. In this report, we used an adenovirus-mediated gene delivery system to express the p202 gene (Ad-p202) in the MDA-MB-468 breast cancer cell line and found that Ad-p202 infection induces growth inhibition as well as sensitizes the otherwise resistant cells to TNF-α-induced apoptosis. Importantly, we demonstrated that Ad-p202 infection alone induces apoptosis, and that requires the activation of caspase-3 for full apoptotic effect. Furthermore, we showed the efficacy of Ad-p202 treatment in both breast and pancreatic cancer xenograft models, and this anti-tumor effect correlated well with p202 protein expression and enhanced apoptosis in tumors. Taken together, our results suggest that Ad-p202 is a potent growth-suppressing, pro-apoptotic agent that not only provides a useful tool to investigate the function of p202, but also can be developed into a therapeutic agent in human cancer gene therapy treatment.

#3970 Icodextrin solution improves adenovirus mediated therapy in intraperitoneal cancer models. Heidrun Engler, Shu Fen Wen, Todd Machemer, Alice E. Harper, Dan Maneval, and Susan E. Conroy. *Canji, Inc, San Diego, CA, and ML Laboratories PLC, St Albans, UK.*

Intraperitoneal (IP) delivery of adenovirus-based therapies is underway for the treatment of ovarian cancer. These therapies may be improved by formulations designed to optimize peritoneal distribution and reduce adhesions. Our objective was to evaluate an icodextrin solution, determining efficacy of adenovirus-mediated therapies delivered into the peritoneal cavity. In solution, icodextrin (an alpha 1-4 linked glucose polymer) exhibits prolonged peritoneal cavity residence times in man and, in an animal model, prevents adhesions resulting from IP bleomycin or adriamycin chemotherapy. Tumors were established in female nude mice by injecting PC-3 cells (human prostate) or MDA-H2774 cells (human ovarian cancer) IP. Therapy consisted either of a replication deficient adenovirus carrying the p53 tumor suppressor gene (rAd-p53), or an oncolytic conditionally replication competent adenovirus (01/PEME). To compare the impact of the vehicle on intraperitoneal delivery, viruses were formulated in an icodextrin solution or in PBS. Animals received four treatments, twice weekly of rAd-p53 (1E10 particles/dose), 01/PEME (5E7 particles/dose), or appropriate controls. rAd-p53 treatment was started nine days (PC-3 model) or two days (2774 model) after cell injection; 01/PEME-treatment (2774 model) was started seven days after cell injection. After treatment, mice were caged randomly and monitored daily for body weight loss (PC-3) or ascites (2774). Moribund animals were sacrificed and survival was plotted on a Kaplan Meier survival curve. rAd-p53 treatment in both cancer models resulted in prolonged survival ($p<0.05$) versus controls with the icodextrin formulation. Enhanced effects of the icodextrin formulation were also observed with 01/PEME. With the 01/PEME virus, mean survival was 28.5 days (vehicle only), 41 days (01/PEME in PBS), >80 days (01/PEME in icodextrin). These findings suggest that an icodextrin formulation can improve intraperitoneal delivery of therapeutic replication deficient and replication competent adenoviruses, resulting in prolonged survival in two different intraperitoneal models of human cancer.

#3971 Selective expression of the GFP-TRAIL fusion gene delivered by an adenoviral vector induces apoptosis in human breast cancer cells and hepatocarcinoma cells. Tongyu Lin, Jian Gu, Lidong Zhang, Xuefeng Huang, Yinhua Yu, Steven A. Curley, Kelly K. Hunt, and Bingliang Fang. *M.D. Anderson Cancer Center, Houston, TX.*

Direct transfer of the human tumor necrosis factor related apoptosis-inducing ligand (TRAIL) gene or the green fluorescent protein (GFP)/TRAIL fusion gene can induce apoptosis and the apoptotic bystander effect in various human cancer cells. However, expression of membrane-bound TRAIL from the full-length coding sequence of the TRAIL gene may cause toxic effects in some susceptible normal cells. On the other hand, the human telomerase reverse transcriptase (hTERT) promoter is active in most malignant cells but silent in normal somatic tissues. Therefore, expression of the TRAIL gene from the hTERT promoter may have both the TRAIL genes therapeutic efficacy and the hTERT promoters target specificity. To understand the therapeutic value of the TRAIL gene expressed from the hTERT promoter, we recently constructed an adenoviral vector expressing GFP-TRAIL fusion protein and driven by the hTERT promoter (Ad/gTRAIL). Transgene expression and apoptosis induction were then tested in a variety of human breast cancer cell lines and human hepatocellular carcinoma cell lines. Our results showed that treatment with Ad/gTRAIL elicited high levels of transgene expression and apoptosis in malignant breast cells and in hepatoma cells but not in normal human mammary cells. Furthermore, both adryamycin-sensitive and -resistant MB-231 breast cancer cells are sensitive to the treatment of Ad/gTRAIL. These results suggest that the adenovirus encoding the GFP/TRAIL gene driven by the hTERT promoter has potential application in cancer therapy.

#3972 Biological and immunological properties of helper-dependent adenovirus (HD-Adv) when used to transduce dendritic cells. Airi Harui, Sylvia M. Kiertscher, Qingwen Cheng, Michael D. Roth, Kohnosuke Mitani, and Saroj K. Basak. *University of California Los Angeles, Los Angeles, CA.*

Replication deficient adenovirus (AdV) is an attractive vector system for delivering genes for immunotherapy. Two main replication deficient AdV systems, FG-Ad virus (E-1/E-3 lacking, first generation) and HD-AdV (fully deleted/gutted helper dependent AdV) have been developed for gene therapy. The FG-AdV still expresses viral genes capable of stimulating immune responses and contributing to the rapid clearance of virus-infected cells from the host. The second generation HD-AdV lacks all viral genes and has been reported to produce long-term gene expression in animal models. Other than this, there is limited information about the effective use of HD-AdV for gene therapy or the nature of its interaction with host immunity. One of the best approaches for inducing immunity has been to vaccinate with antigen-presenting dendritic cells (DC) that have been transduced with AdV in order to express a target antigen. In this report, we examine both the biological and immunological properties of FG-AdV and HD-AdV when used to transduce DC for genetic immunotherapy. Using an *in vitro* model, we found that both FG-AdV and HD-AdV can be used to express transgenes in human and mouse DC, but that expression is 10 to 100 fold higher following infection with HD-AdV. Both vectors exhibit similar transduction efficiencies, with enhanced expression in HD-ADV-transduced cells resulting from higher levels of transgene-specific mRNA. We further observed that transduction of DC with a combination of HD-AdV and FG-AdV resulted in a synergistic enhancement of transgene expression. This enhancement was knocked-out by UV-irradiation of the FG-AdV, indicating that gene products synthesized by the FG-AdV work in trans to drive expression from the HD-AdV transgene. When these AdV were tested for their ability to induce cell mediated immune responses *in vitro*, DC transduced with either the HD-AdV alone, the FG-AdV alone, or the combination of FG-AdV/HD-AdV, stimulated the proloiferation of AdV-specific and nonspecific T cells to the same degree. UV irradiation had no effect on the immune response, suggesting that it was directed primarily against the preformed viral capsid. These features suggest that HD-AdV, or the combination of HD-AdV/FG-ADV, might provide valuable tools for obtaining high level of transgene expression in DC, but might still elicit anti-viral (capsid) immunity. The study was supported by grants from the Tobacco-Related Research Program of California (# 10KT-0086) and the American Lung Association of California to SKB.

#3973 The therapeutic effect of double-mutated adenovirus, AxdAdB-3, targeting abnormal genetic pathways in pancreatic cancer. Hisashi Abe, Fuyuhiko Motoi, Makoto Sunamura, Shinichi Egawa, Kazunori Takeda, Hirofumi Hamada, and Seiki Matsuno. *Tohoku University, Sendai City, Japan, and Sapporo Medical University, Sapporo, Japan.*

(Aim and Background) Pancreatic cancer has genetic abnormalities frequently including p53, p16/MTS1 and SMAD4. A replication-selective adenovirus (RSA) that preferentially destroys cancer cells was first described in 1996. This adenovirus lacking E1B55K can replicate selectively in tumor cells without p53 normal function and kills cells. An adenoviral E1A protein binds to RB to force the quiescent cell into S-phase. Many cancers have the mutations of RB/E2F/p16 pathway, leading the loss of control in cell cycle. It is also reported that the adenovirus with a mutation of E1A incapable of binding to RB replicates in the tumor cells with an unregulated cell cycle, but cannot replicate in normal cells possessing tightly regulation in cell cycle. We constructed a double-mutated RSA (AxdAdB-3) that has a mutation in RB-binding motif and a deletion of E1B55K and evaluate its therapeutic effect on pancreatic cancers. (Method) We used 8 pancreatic cancer cell lines and human fibroblast cell. 1) *In vitro* cytopathic effect was

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Dhanesuan et al., Breast cancer research and treatment (netherlands), Sept. 2002, 75, 1, p73-85.

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Podhajcer et al., Breast 5, 1, p13-20, 1996.

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212.17

Genotoxic effects of industrial wastewater effluents in Vicia faba

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In this study the effect of benzene, sodium dichromate, and dichloromethane which are considered to be the industrial priority pollutants (Rank and Nielsen, 1994), on the genetic endpoints were determined. Vicia faba (Broad bean) seedlings were treated with the chemical in a randomized block design with four replications. Two concentrations for each of the test chemicals were selected. 100 μM and 1000 μM of benzene, 50 μM and 250 μM of sodium dichromate, and 175 μM and 1750 μM of dichloromethane were used as concentrations. Lateral roots were treated with test chemicals for two hours in the dark. The seedlings were then transferred into distilled water for 24 hours recovery period. Root tips were harvested after two hours treatment with 0.05% colchicine. Root tip squashes were stained with feulgen stain and data was collected for mitotic index and chromosomal aberrations. Results showed a significant decrease in the mitotic index and increase in chromosomal aberrations with all the test chemicals when compared with control. The decrease in mitotic index and increase in chromosomal aberrations were dose dependent. (Supported by Department of Education grant # PO31B44000-95)

212.18

Effects of Tyrosine Hydroxylase Inhibition and Gene Mutations Upon Locomotor Activity in *Drosophila*: A Study in Functional Genomics

Robert G. Pendleton, Aseel Rasheed, Thomas Sardina, Ralph Hillman. Temple University, Philadelphia, PA 19122

The brain of the adult fruit fly, *Drosophila melanogaster*, contains tyrosine hydroxylase, the rate limiting enzyme required for catecholamine biosynthesis. As a consequence catecholamines, principally dopamine are also present. We have found that pharmacological inhibition of tyrosine hydroxylase with α methyl-p-tyrosine results in a dose related inhibition of locomotor activity in adult organisms. Similar results were found with reserpine, a well known inhibitor of catecholamine uptake into storage granules, and could be prevented in each case by the concomitant administration of L-DOPA. The single copy gene coding for tyrosine hydroxylase in *Drosophila* is *pale* (*ple*). Both null and temperature sensitive loss of function mutant alleles of *pale* are known which are recessive embryonic lethals. Null mutant heterozygous flies have normal locomotor activity demonstrating that only a single dose of the wild type form of this gene is required to support normal function. Both hemizygous and homozygous temperature sensitive *pale* mutants (ple^{ts}) also show normal locomotor activity at the permissive temperature for this mutant allele (18°C) which progressively declines as the temperature is increased to its restrictive level (29°C). These abnormal locomotor effects were preventable by L-DOPA. Thus the effects on locomotor activity resulting from the pharmacological inhibition of catecholamine synthesis or storage are the same as those resulting from lack of tyrosine hydroxylase expression. These findings indicate that brain catecholamine loss decreases locomotor activity in the fly as it does in mammals. They also demonstrate the ability of functional genomic studies to mimic that of pharmacological inhibition of enzyme function or other similar processes.

PROSTATE CANCER (214.1-214.4)

214.1

DIFFERENTIAL EXPRESSION OF HEPATOCYTE GROWTH FACTOR AND C-MET IN PROSTATE CANCER AMOUNT CAUCASIAN AMERICAN VS. AFRICAN AMERICAN PATIENTS

Sharon Collins Presnell, Kristen Borchert, Chris Gregory, Susan Maygarden, Gary Smith, James Mohler: University of North Carolina, CB#7525, BBB, Chapel Hill, NC 27599-7525

African-American (AA) men are 2-3 times more likely to die from prostate cancer (CaP) than Caucasian-American (CA) men, and epidemiological studies have shown that AA males with non-organ-confined CaP have higher PSA levels and a higher incidence of recurrence than CA males with non-organ-confined CaP. We evaluated expression of Hepatocyte Growth Factor (HGF), its' receptor (c-Met), and Androgen Receptor (AR) in prostate cancer specimens collected from 10 AA and 10 CA CaP patients at the time of prostatectomy. All androgen-dependent (AD) specimens were Gleason score 6-7, and all androgen-independent (AI) specimens were Gleason score 8-10. In the AD specimens, there was a 4-fold increase in expression of c-Met protein in AA compared to CA patients, while expression of HGF and AR was not significantly different. The AI samples from AA patients demonstrated a 6-fold increase in c-Met and HGF in comparison to AI samples from CA patients, while AR expression was comparable between the two groups. Immunohistochemical analysis of the specimens demonstrated that c-Met expression was greater in the prostate epithelial cells from AA vs. CA, even in benign glands with no evidence of neoplasia. These data suggest that prostatic c-Met expression is higher in AA males than CA males, and that androgen-independent CaP arising in AA males is more likely to overexpress HGF as well. Given the established association of HGF and c-Met with the progression of CaP, these results provide new insight into potential mechanisms of racial bias in prostate cancer.

214.2

INCREASED EXPRESSION OF HEPATOCYTE GROWTH FACTOR, C-MET, AND ANDROGEN RECEPTOR IS ASSOCIATED WITH THE TRANSITION FROM ANDROGEN-DEPENDENT TO ANDROGEN-INDEPENDENT PROSTATE CANCER

Sharon Collins Presnell, Kristen Borchert, Chris Gregory, Susan Maygarden, James Mohler: University of North Carolina, CB#7525 Brinkhous-Bullitt Bldg., Chapel Hill, NC 27599-7525

We have shown previously that Hepatocyte Growth Factor (HGF) is capable of ligand-independent activation of the androgen receptor (AR) in prostate epithelial tumor cell lines in vitro. The goal of the present study was to evaluate expression of HGF, its' cognate receptor (c-Met), and AR in androgen-dependent (AD) and androgen-independent (AI) human prostate cancer (CaP) specimens. Expression levels of HGF, c-Met, and AR proteins were evaluated by immunohistochemistry and western blot analysis in 10 cases each of benign prostatic hyperplasia (BPH), AD CaP, and AI CaP. AR expression was slightly but significantly elevated in 5/10 AD CaPs, but was greatly elevated in 7/10 AI CaPs. HGF was expressed by 2/10 AD CaPs, but was elevated more consistently in AI CaP (5/10). Full-length c-Met protein was overexpressed in 7/10 AD CaPs in comparison to BPH, but was elevated in only 3/10 AI CaPs. Interestingly, the decrease in full-length c-Met among the AI CaPs was accompanied by acquisition of expression of an 85 kDa N-terminal truncated variant of c-Met that may represent a constitutively active receptor. Combined, these data suggest that the transition from androgen-dependent to androgen-independent prostate cancer involves upregulation of AR and HGF, along with acquired expression of a truncated (and possibly active) HGF receptor. Given the observations that: 1) AR is involved in AI CaP; 2) HGF can activate AR in the absence of testicular androgens; 3) AI CaP emerges in an environment lacking testicular androgens; and 4) removal of testicular androgens results in increased expression of both HGF and c-Met, it is plausible that both HGF and c-Met play a role in the progression of prostate cancer by providing an alternative mechanism for driving AR-dependent processes in an androgen-independent environment.

214.3

Stroma-Epithelial Interaction in Prostate Cancer as the Basis for Molecular Co-Targeting with Adenoviral Vectors

Leland W.K. Chung, Andy Law, Chia-Ling Hsieh, Shigeji Matsubara, Hong Rhee: University of Virginia, U. Virginia Health Sciences Center, Charlottesville, VA 22908

Reciprocal cell interaction between prostate cancer and bone or prostate stromal cells demonstrated the importance of tumor and microenvironment interaction, which regulates local tumor growth, and its acquisition of androgen-independence and metastatic potential. This study documented that prostate cancer and bone or prostate stromal cells when co-cultured under 3-D microgravity simulated growth conditions underwent non-random permanent phenotypic and genotypic changes in both tumor epithelial and bone stromal cellular compartments. These results support the role of cellular interaction and prostate carcinogenesis. Using lineage-derived LNCaP cell lines as a model for the evaluation of multi-step prostate carcinogenesis, we observed that prostate cancer cells that have acquired the ability to metastasize to the skeleton exhibit osteo-mimetic properties. By employing this cellular interaction model, we have developed a series of tissue-specific and tumor-restrictive promoters, osteocalcin, bone sialoprotein, and osteonectin, which have a unique ability to target prostate cancer and prostate and bone stromal cells in tumors. This strategy of co-targeting has been applied in both pre-clinical and/or clinical settings. We have constructed and tested replication-defective adenoviruses to treat both localized and metastatic prostate cancers. This strategy is being expanded presently to the use of replication competent adenoviral vectors for the treatment of prostate cancer skeletal metastasis.

214.4

Suppression of Prostate Tumor Xenograft Growth Following Localized Treatment with TRAIL/Apo-2L Expressing Recombinant Adenovirus.

Thomas S. Griffith, Richard D. Anderson, Beverly L. Davidson, Elizabeth L. Broghammer, Richard D. Williams, Timothy L. Ratliff: University of Iowa, Urology, 200 Hawkins Dr., Iowa City, IA 52242

TRAIL (TNF-related apoptosis-inducing ligand)/Apo-2L is a member of the TNF superfamily that induces apoptosis in a variety of tumor cell types. Numerous studies have demonstrated that recombinant, soluble forms of TRAIL are potent mediators of tumor cell apoptosis in vitro and in vivo, while demonstrating minimal cytotoxicity toward normal tissues. One potential disadvantage to previous in vivo protocols was the need for large quantities of TRAIL to significantly inhibit tumor outgrowth. In the current study, a replication-deficient adenovirus was engineered to encode the human TRAIL gene (Ad5-TRAIL) as an alternative to recombinant, soluble TRAIL protein. The results clearly show that TRAIL-sensitive prostate tumor cell targets infected with Ad5-TRAIL rapidly produce and express TRAIL protein, and subsequently undergo apoptotic death. This activity is limited to TRAIL-sensitive tumor cells, as normal prostate epithelial cells or TRAIL-resistant tumor cells are not killed following Ad5-TRAIL infection. Furthermore, in vivo administration of Ad5-TRAIL at the site of tumor implantation suppressed human prostate tumor xenograft outgrowth in immunodeficient SCID mice. Histologic examination of prostate tumors treated locally with Ad5-TRAIL displayed areas of apoptosis within 24 h of injection. These results further define the use of a recombinant adenovirus encoding TRAIL (Ad5-TRAIL) as a novel anti-tumor therapeutic, and demonstrate its potential use as a means for treating prostate, as well as other solid, tumors in vivo.

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Podhajcer et al., Breast 5, 1, p13-20, 1996.

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TOXICOLOGY AND APPLIED PHARMACOLOGY 116, 240–247 (1992)

Lead Inhibits Secretion of Osteonectin/SPARC without Significantly Altering Collagen or Hsp47 Production in Osteoblast-like ROS 17/2.8 Cells

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Lead Inhibits Secretion of Osteonectin/SPARC without Significantly Altering Collagen or Hsp47 Production in Osteoblast-like ROS 17/2.8 Cells. SAUK, J. J., SMITH, T., SILBERGELD, E. K., FOWLER, B. A., AND SOMERMAN, M. J. (1992). *Toxicol. Appl. Pharmacol.* 116, 240–247.

In an effort to better understand the consequences of lead (Pb^{2+}) on skeletal growth, the effects of Pb^{2+} were investigated using ROS 17/2.8 bone-like cells *in vitro*. These studies revealed that Pb^{2+} (4.5×10^{-6} M – 4.5×10^{-7} M) has little or no effect on cell shape except when added immediately following seeding of the cells. However, proliferation of ROS cells was inhibited, in the absence of serum, at concentrations of 4.5×10^{-6} M Pb^{2+}. Protein production was generally increased, however, the major structural protein of bone, type I collagen, production was only slightly altered. Following treatment of ROS cells with Pb^{2+}, intracellular levels of the calcium-binding protein osteonectin/SPARC were increased. Osteonectin/SPARC secretion into the media was delayed or inhibited. Coincident with retention of osteonectin/SPARC there was a decrease in the levels of osteonectin/SPARC mRNA as determined by Northern analysis. These studies suggest that processes associated with osteonectin/SPARC translation and secretion are sensitive to Pb^{2+}.

Lead (Pb^{2+}) is a toxic substance that is known to impair skeletal growth even at levels of exposure for the general population (0.75 μM) (Mooty *et al.*, 1975; Angle and Huseman, 1989; Shukla *et al.*, 1989; Schwartz *et al.*, 1986; Frisanch and Ryan, 1991). There are several indications that Pb^{2+} by its interactions with calcium (Ca^{2+}) may perturb many normal calcium regulatory processes, e.g., its action as a second messenger, interactions with calcium binding and other extracellular proteins associated with mineralized tissues and subcellular functions at the level of the mitochondria, synaptosomes, and membrane vesicles. Furthermore, lead is known to affect protein synthesis (Shelton *et al.*, 1986), particularly by increasing proteins that constitute nuclear and cytoplasmic inclusion bodies (Moore *et al.*, 1973; Richter *et al.*, 1968; Choie *et al.*, 1975; McLachlin *et al.*, 1980; Shelton and Egle, 1982), two glucose regulated proteins (Shelton *et al.*, 1986) and by deminishing the levels of the bone related protein, osteocalcin (Angle *et al.*, 1990; Long *et al.*, 1989, 1990).

In addition to osteocalcin, there is increasing evidence that other noncollagenous proteins associated with mineralized tissues play a critical role in the regulation of osteogenesis. This has led to increased research emphasis on purifying, characterizing, and identifying the role of these proteins in the mineralization process (for reviews see Butler 1985; Fisher *et al.*, 1987; Glimcher and Lian, 1989). Many of these proteins are acidic and are known to bind both Ca^{2+} and hydroxylapatite, suggesting a role for them in the initial phases of mineralization. Also, some bone related proteins such as osteonectin/SPARC (secreted protein, acid and rich in cysteine) mRNA have been localized to areas of active tissue morphogenesis and are expressed in cells and tissues that exhibit high rates of turnover, secretion, and remodeling (Funk and Sage, 1991). In the studies reported here we seek to determine if Pb^{2+} alters the characteristics of ROS 17/2.8 bone-like cells *in vitro*. Also, this study determines whether Pb^{2+} affects the production of two recognized bone matrix components, collagen and osteonectin/SPARC. Furthermore, we examine the effect of Pb^{2+} on Hsp47, a resident endoplasmic reticulum protein of cells producing type I collagen (Nakai *et al.*, 1990; Sauk *et al.*, 1990b).

MATERIALS AND METHODS

Cell culture. Cloned bone cell lines (ROS 17/2.8) derived from ACI9... rat osteosarcoma were used. These cells have the advantage that their osteoblastic-like phenotypic characteristics are maintained *in vitro* (Majeska *et al.*, 1980). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 1.16 g/l glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin and [illegible] fetal bovine serum (FBS). Lead glutamate was prepared by preparing a stock solution containing $4.5 \times 10^{-?}$ M lead nitrate (Sigma) and [illegible] M glutamic acid in water.

Proliferation rate. [illegible] ...ate the effects of lead on cell proliferation ROS 17/2.8 cells [illegible] ...d into tissue culture wells (35 mm Falcon

$\approx 8.0 \times 10^3$ cells/well with DMEM supplemented with 10% FBS and antibiotic and incubated overnight at 37°C. On the following day, the medium was removed from each well and medium added as follows: (a) DMEM + 10% FBS + glutamic acid; (b) DMEM + 2% FBS + glutamic acid; (c) DMEM + 0% FBS + glutamic acid; (d) DMEM + 2% FBS + lead glutamate; and (e) DMEM + 0% FBS + lead glutamate. The effects of lead glutamate on cell proliferation were evaluated at three different concentrations, 4.5×10^{-5} M, 4.5×10^{-6} M and 4.5×10^{-7} M. Cell proliferation, on Day 2, Day 4, and Day 6 after seeding, was determined by trypsinizing cells and counting electronically using a Coulter counter.

Determination of total protein and collagen production. The methods for labeling cells have been reported previously (Somerman *et al.*, 1987). Briefly, 1.0×10^4 cells per test well (Falcon, 35-mm wells) were seeded in wells in DMEM with 10% FBS and antibiotics. After cells attached and spread, medium was removed and cells were incubated for 48 hr in serum-free DMEM with 20 μCi/ml L-5-[^{3}H]proline and (a) glutamic acid (control) or (b) 4.5×10^{-6} M lead glutamate. Media and cell samples were then dialyzed extensively against cold buffer (pH 7.4, 0.05 M Tris–HCl, 0.2 M NaCl, 0.005 M $CaCl_2$). Following dialysis, aliquots of these samples were counted in a liquid scintillation spectrometer (Packard Tri Carb 460C) in Aquasol-2 (New England Nuclear (NEN) Boston, MA). These counts represented total non-dialyzable radioactive material and were taken as a measure of total protein production.

To estimate collagen production, an aliquot of the above dialyzed material was reacted for 6 hr at 37°C with chromatographically purified bacterial collagenase (Form III, Advance Biofactures) according to methods of Peterkofsky and Diegelmann (1971). Undigested proteins were precipitated with 5% trichloroacetic acid/0.5% tannic acid and samples were centrifuged. An aliquot of the supernatant was counted in Aquasol-2 (NEN) with a liquid scintillation counter. To provide a background value for the collagenase assays, additional aliquots of dialyzate were treated as above except that bacterial collagenase was excluded from these samples. The resulting backgrounds were subtracted from the collagenase-treated series to yield a value which represented collagen production expressed as collagenase digestible protein.

To determine the effects of lead on protein synthesis, cells were incubated with ^{35}S-methionine and 4.5×10^{-6} M lead glutamate for 20 min and then cell and media were harvested separately, as described above. Nonradioactive wells were run in parallel with these experiments to obtain total number of cells/well at time of harvest. The cells were harvested with 0.08% trypsin/0.04% EDTA and total cell number per well was determined electronically by Coulter counter. All assays were run in triplicate and data expressed as CPM $\times 10^{-3}/10^6$ cells.

Gel electrophoresis. For polyacrylamide gel electrophoresis (PAGE), cells were seeded and exposed to 4.5×10^{-6} M lead glutamate and radioactively labeled exactly as described above. Media and cell samples were dialyzed extensively against distilled water and lyophilized. Comparable cell and media samples, of control and lead-treated cells, were dissolved in gel buffer. Thus, any variation in protein profiles among samples is based on altered protein production/well during the labeling period. Following reduction with mercaptoethanol, the samples were analyzed by gel electrophoresis on SDS 4–20% polyacrylamide gradient slab gels according to the method of Laemmli (1970). The gels were fixed, dried, and autoradiographed using the method of Bonner and Laskey (1974).

Immunoblotting. For Western blots, 3×10^6 cells were plated and SDS-PAGE was performed as described above and proteins were immediately electrotransferred to nitrocellulose paper (Towbin *et al.*, 1979). The paper was blocked with 3% bovine serum albumin (BSA) in 10 mM Tris–HCl pH 7.4, 0.9 M NaCl (TBS) for 1 hr and then in TBS/BSA with 2% normal goat serum (NGS–GIBCO). Antiserum, rabbit-anti-bovine osteonectin (gift of L Fisher NIDR/NIH), or preimmune serum were diluted 1:2000 in the same solution and incubated with gentle shaking overnight. The nitrocellulose paper was then rinsed three times for 5 min in TBS/Tween. The secondary antibody, affinity-purified goat-anti-rabbit IgG (Fc) horseradish peroxidase (Kirkegaard & Perry Labs, Inc., Gaithersburg, MD) was diluted to 0.9 μg/ml and incubated with the paper for 2 hr. Washing between steps was performed three times for 30 min with 50 mM Tris–HCl, 0.9 M NaCl, 0.05% Tween, pH 7.4. Staining was accomplished with 4-chloro-naphthol. For Hsp47, polyclonal rabbit antibodies were prepared against a 22-mer peptide corresponding to the N-terminal sequence of chick Hsp47 (Hirayoshi *et al.*, 1991) that was conjugated to Keyhole limpit hemocyanin (Sauk *et al.*, 1990b).

Immunoprecipitation. Cell layers having equal cell numbers were homogenized in 2× immunoprecipitation buffer (0.2 M Tris–HCl, 0.3 M NaCl, 2% Triton X-100, 2% deoxycholate, 0.2% SDS, pH 7.2) and diluted with cold distilled water to 1×, while the culture media was diluted 3:1 with 4× immunoprecipitation buffer. The homogenates and media were then centrifuged for 5 min at 10,000g in an Eppendorf centrifuge and a 50-μl sample of the radiolabeled supernatant was added to a mixture of GammaBind G-Agarose (Genex, Gaithersburg, MD) and antibody in PBS–azide. The samples were then incubated at 4°C with constant shaking and then centrifuged at 10,000g for 10 min. The resulting immunoprecipitate pellets were then washed twice with PBS–azide. The final pellets were suspended in 2× gel electrophoresis sample buffer, heated for 10 min at 90°C, and then centrifuged to remove GammaBind G-agarose. Samples of the supernatants were counted in a scintillation counter and another sample analyzed by PAGE and autoradiography as described above. Autoradiograms were densitometrically scanned and data were reported as ratio of treated to control samples.

Northern analysis. Total RNA from 4×10^6 ROS 17/2.8 cells was solubilized in 500 μl 4 M guanidinium thiocyanate containing 25 mM sodium acetate (pH 5.0) and 100 mM β-mercaptoethanol, transferred to an Eppendorf tube containing 20-μg of calf liver tRNA, and precipitated by addition of 450 μl of 100% ethanol. RNA pellets were reprecipitated in the presence of 80% ethanol containing 100 mM sodium acetate (pH 5.0) at −20°C, and equal amounts of RNA were immediately loaded onto Northerns. RNA was transferred to nitrocellulose and bound to the paper by baking at 80°C for 2 hr *in vacuo*. A 1.5-kb *Eco*RI restriction fragment of a bovine osteonectin cDNA (Sauk *et al.*, 1991) was radiolabeled by nick translation (Amersham Kit) using α-[P^{32}] dCTP and hybridized to filter bound RNA. Hybridization was carried out at 37°C in a mixture of 40% formamide, 0.1 g/ml dextran sulfate, 5× SSC (74 mM sodium citrate, 750 mM sodium chloride), 10 mM Tris (pH 7.5), 4× Denhardt's solution, and 0.1 mg/ml denatured salmon sperm DNA. The filters were washed in a solution of 2× SSC, 0.1% SDS three times for 15 min at 25°C and autofluorographed. Values of the osteonectin/SPARC mRNA (arbitrary densitometric units) were normalized to the amount of ribosomal RNA.

Attachment assay. The attachment assay utilized is similar to that of Klebe (1974). Uncoated polystyrene bacteriological 24-well plates (bacteriological petri dishes, 25820 special Corning) containing the specific factor being evaluated, i.e., fibronectin (10 μg/ml, positive control) or lead glutamate at a concentration of 4.5×10^{-6} M and 4.5×10^{-7} M, were preincubated for 1 hr at 37°C in 0.4 ml DMEM containing 1 mg/ml bovine serum albumin (BSA, Fraction V Miles Laboratories, Inc.). Control wells had DMEM plus BSA. Following the conditioning period ROS 17/2.8 cells, at a concentration of 3×10^4 cells in 0.1 ml of DMEM/BSA were added to the wells. Incubation was continued for an additional 90 min and wells were rinsed with Hanks' balanced salt solution to remove unattached cells. Photographs, as needed, were taken at this time to document cell spreading. Cells remaining were removed enzymatically with 0.08% trypsin/0.04% EDTA and counted electronically using a Coulter counter.

Cell spreading. Cell spreading was determined by photographing six representative microscopic fields at ×10. The photographic images were then captured using an ELMO visual presenter (EV-368) and the shape of the resulting cells was determined following image analyses using NIH Image (1.40) software.

Statistical analyses. All experiments consisted of at least triplicate determinations. The statistical significance of differences in effects of Pb^{2+} on cells was determined by analysis of variance and Duncan's multiple range test.

RESULTS

Initially, lead acetate and lead glutamate were both evaluated for use in these *in vitro* assays. Lead acetate added to DMEM, at comparable doses to lead glutamate (10^{-5} M), resulted in the formation of an immediate visible precipitate. No visible precipitate was noted in lead glutamate solutions, including the 4.5×10^{-2} M stock solution. Thus, lead glutamate was selected for use in determining the effects of Pb^{2+} on bone cells, *in vitro*.

For proliferation studies cells were exposed to three concentrations of Pb^{2+}, 4.5×10^{-5} M, 4.5×10^{-6} M, and 4.5×10^{-7} M in DMEM/2% FBS or DMEM/0% FBS. After 1 day exposure of cells to 4.5×10^{-5} M Pb^{2+} in DMEM/0% FBS a visible precipitate was noted. By Day 4 a similar precipitate was noted in cells exposed to 10^{-5} M Pb^{2+} in DMEM/2% FBS and a decrease in cell proliferation was noted compared to controls (Fig. 1). Concentrations of Pb^{2+} less than 4.5×10^{-5} M had no effect on cell proliferation in the presence of serum. However, in the absence of serum, 4.5×10^{-7} M Pb^{2+} increased cell proliferation at Day 4 while, 4.5×10^{-6} M Pb^{2+} inhibited proliferation, but not until Day 6.

The *in vitro* attachment assay revealed that Pb^{2+} (4.5×10^{-6} M–4.5×10^{-8} M) did not affect cell attachment. Furthermore, fibronectin attached cells were not displaced by the addition of Pb^{2+} to the media (Table 1). However, if Pb^{2+} (4.5×10^{-6} M) was incorporated into the medium of cells within the first 3 hr, 92 ± 3% the cells that attached did not go on to spread (Fig. 2A). This nonspread condition persisted for up to 4 days, after which the experiment was terminated.

TABLE 1
Effect of Lead on Attachment of ROS 17/2.8 Cells

Treatment	Number of cells attached/well	% Attached of applied
Control	960 ± 236	3.2 ± 0.8
FN	15407 ± 1345[a]	51.4 ± 4.5
Na glutamate (0.5×10^{-5} M)	1009 ± 130	3.4 ± 0.4
Pb glutamate (4.5×10^{-5} M)	1073 ± 301	3.6 ± 1.0
Pb glutamate (4.5×10^{-6} M)	1062 ± 415	3.5 ± 1.4
Pb glutamate (4.5×10^{-7} M)	1083 ± 108	3.6 ± 0.4
Na glutamate (5.0×10^{-5} M) + FN	16622 ± 2043[a]	55.4 ± 6.8
Pb glutamate (4.5×10^{-5} M) + FN	14529 ± 1366[a]	48.4 ± 4.5
Pb glutamate (4.5×10^{-6} M) + FN	14220 ± 1038[a]	47.4 ± 3.5
Pb glutamate (4.5×10^{-7} M) + FN	15189 ± 2503[a]	50.6 ± 8.3

[a] $p < 0.05$ when compared with control wells.

Note. FN, fibronectin. All agents were evaluated in triplicate and data expressed as number of cells attached ± SD (3.0×10^4 cells per well were applied). There were day to day variations in the number of cells applied. For clarity and consistency results are expressed as the number of cells attached per well ± SD.

If the cells were allowed to attach and spread overnight, Pb^{2+} added to the medium did not affect spreading, consequently 85 ± 9% of Pb^{2+} treated and 87 ± 6% of control cells were noted to spread (Fig. 2).

Based on proliferation and attachment results, Pb^{2+} at 4.5×10^{-6} M was added 24 hr after plating and used in assays designed to determine the effect of Pb^{2+} on biosynthetic ac-

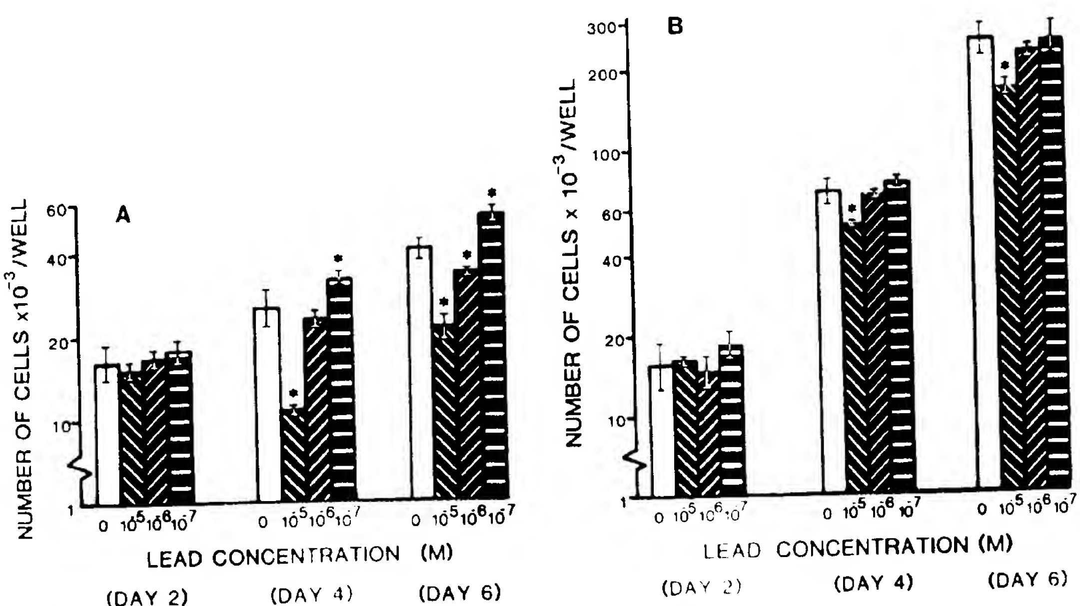

FIG. 1. The effect of lead glutamate (4.5×10^{-5}–4.5×10^{-7} M) in the absence (A) and presence (B) of 2% fetal bovine serum on the proliferation of ROS 17/2.8 cells. Data represent the mean of three determinations ± the standard deviation (I). * Indicates significantly ($p = 0.05$) different from control by ANOVA.

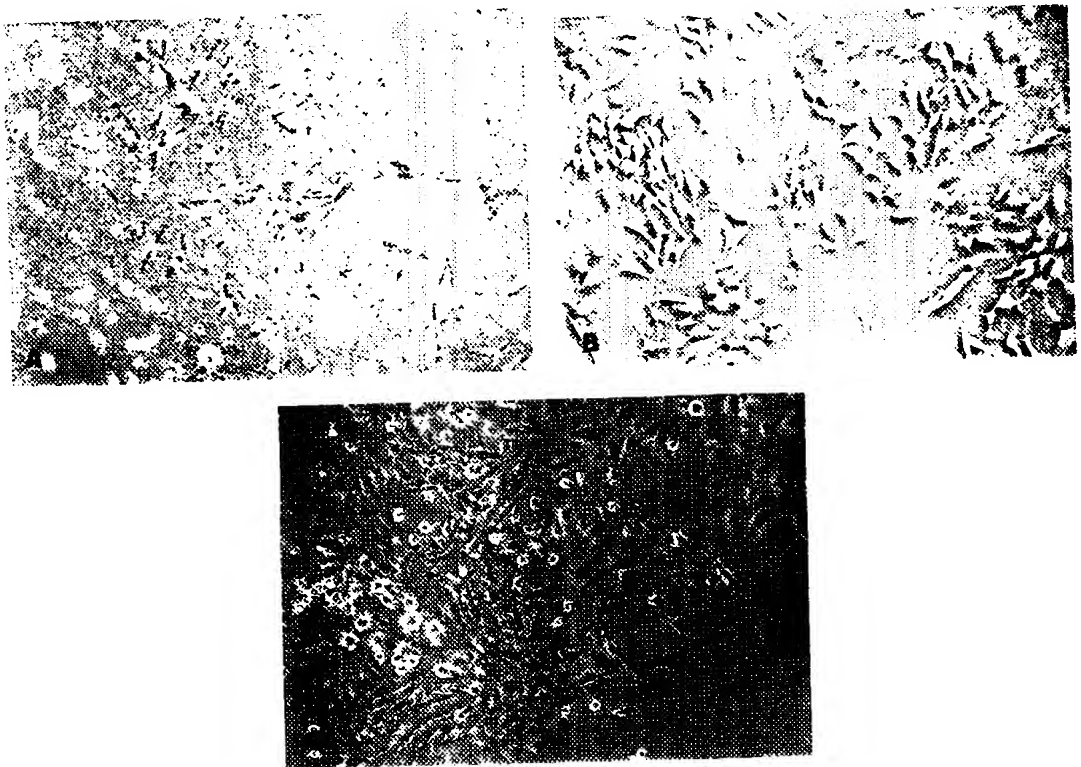

FIG. 2. (A) Attached and rounded (unspread) ROS 17/2.8 cells treated with 4.5×10^{-6} M Pb^{2+} 3 hr after plating. Cells were then maintained for an additional 21 hr in a humidified atmosphere as described under Materials and Methods. (B) Attached and spread ROS 17/2.8 cells that were treated with 4.5×10^{-6} M Pb^{2+} 12 hr after plating. Cells were then maintained for an additional 12 hr in a humidified atmosphere as described under Materials and Methods. (C) Control cells grown for 24 hr as described under Materials and Methods. Results represent the mean of four separate experiments.

tivity of ROS 17/2.8 cells, *in vitro*. Both media and cells exposed to Pb^{2+} for 48 hr exhibited a significant increase in total protein production/cell, approximately 30 and 40%, respectively, when compared to control. In contrast, no significant increase in collagen production was observed (Table 2). To determine whether Pb^{2+} had a short-term effect on cellular proteins (i.e., protein synthesis) ROS 17/2.8 cells were exposed to 4.5×10^{-6} M Pb^{2+} for 20 min and radio-

TABLE 2
Effect of Lead Glutamate on Protein and Collagen Production by ROS 17/2.8 Cells

	Total protein production (CPM × 10^{-3})	Collagen production (CPM × [illegible])
Cell		
Control	708 ± 86	6[illegible] ± 15
Lead	990 ± 78*	9[illegible] ± 33
Media		
Control	540 ± 42	15[illegible] ± 17
Lead	698 ± 61*	[illegible] ± 25

Note. Cells labeled with [^{3}H]proline were exposed to lead glutamate for 48 hr at a concentration of 4.5×10^{-6} M. Results are expressed as means ± standard deviation. These results were representative of three separate experiments.

* $p <$ [illegible] significantly from control wells.

TABLE 3
Effect of Lead Glutamate on Protein Synthesis by ROS 17/2.8 Cells

	Total protein synthesis (CPM × 10^{-3})
Cell	
Control	357 ± 51
Lead	431 ± 54*
Media	
Control	35 ± 9
Lead	33 ± 5

Note. ^{35}S-methionine and [illegible] M lead glutamate were added to wells for 20 min and then media and cells were harvested separately. Results are expressed as means ± standard deviation. These results were representative of three separate experiments.

* $p < 0.05$ significantly different from control wells.

actively labeled during this time period. As seen in Table 3, Pb^{2+} exposed cells exhibited an increase in protein synthesis, but not in secreted (media) proteins.

To further investigate these changes, we analyzed the effect of varying Pb^{2+} concentrations on cellular levels of Hsp47, a collagen-binding glycoprotein associated with collagen assembly and sorting, and on the acidic bone-related protein, osteonectin/SPARC. Hsp47 levels, as with collagen production, were not altered by Pb^{2+} treatment (Fig. 3). Conversely, cellular levels of osteonectin were noted to be enhanced following Pb^{2+} exposure (Fig. 4). Examination of the media following Pb^{2+} (4.5×10^{-6} M) treatment revealed that osteonectin/SPARC levels were not detectable, by optical density comparison, to that of control cells (Fig. 5). Exposure of ROS 17/2.8 cells to 4.5×10^{-5}–4.5×10^{-7} M Pb^{2+} for a shorter duration (20 min) followed by immunoprecipitation of cell proteins with anti-osteonectin antibodies revealed a dose-dependent decrease in synthesis of osteonectin/SPARC (Fig. 6). Northern analysis of osteonectin mRNA (2.2 kb) showed no differences between Pb^{2+} treated (4.5×10^{-6} M) for 1 hr and control cells. However, if cells were exposed to Pb^{2+} for 24 hr, osteonectin/SPARC mRNA levels were reduced by threefold compared to control cells (Fig. 7).

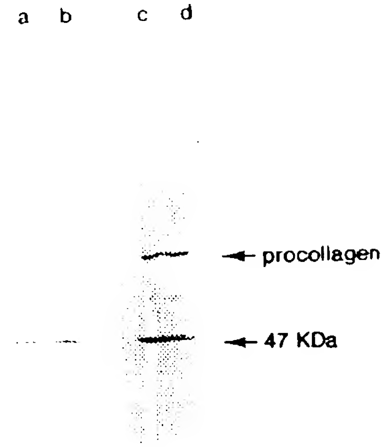

FIG. 3. The effect of 4 hr of 4.5×10^{-6} M Pb^{2+} on Hsp47 in ROS 17/2.8 cells. Lanes a and b represent Western blots utilizing anti-Hsp47 antibodies. Control lane a and Pb^{2+} treated lane b, ROS 17/2.8 cells. Hsp47 was enriched by binding to gelatin–Sepharose (Sauk *et al.*, 1990) prior to PAGE. Lanes c (Control) and d (Pb^{2+} treated) represent autoradiographs of ^{35}S-methionine-labeled (4 hr). ROS 17/2.8 proteins immunoprecipitated with anti-Hsp47 antibodies. In addition to Hsp47, α 1(I) procollagen chains are coimmunoprecipitated with anti-Hsp47 antibodies (Nakai *et al.*, 1990). The optical density of precipitates in Western blots and bands in immunoprecipitate autoradiograms were determined by gel scanner as a component of NIH Image (1.4) software. These analyses revealed relative optical density differences of <0.05 between control and Pb^{2+} treated cells.

DISCUSSION

The data reported here demonstrate that Pb^{2+} decreases the proliferation of bone cells *in vitro* and enhances cellular protein production. The ability of Pb^{2+} to decrease ROS 17/2.8 proliferation is in agreement with similar studies by Long *et al.* (1990). However, at comparable doses (4.5×10^{-6} M) we observe decreased cell growth by Day 6, whereas Long *et al.* did not observe decreased cell growth until Day 9. The reason for these differences may include the experimental design. Although both studies used ROS 17/2.8 cells, we used 0 and 2% serum whereas they used 5% serum. Higher serum levels may lower the concentration of unbound Pb^{2+} available to cells. We also used lead glutamate, while they used lead acetate which is particularly prone to form precipitates (McLachlin *et al.*, 1980).

The importance of cell adhesion in the regulation of many physiological and pathological events including development, platelet aggregation, wound healing, and malignant invasion prompted us to further investigate the effects of Pb^{2+} on bone cell attachment. The adhesion of cells to a substratum, *in vitro* is frequently regulated by calcium dependent, receptor-ligand interactions. Thus a reasonable hypothesis for the observed decreased cell attachment would be that Pb^{2+}, by competing with Ca^{2+} at the cellular membrane, may interfere with receptor–ligand interactions and subsequently cell attachment and spreading. Previous studies reported that marrow cells exposed to Pb^{2+} (1–10 μM Pb^{2+}) for 10 days exhibited decreased adherence to tissue culture dishes (Kowolenko *et al.*, 1989). The ability for ROS 17/2.8 cells to attach, but not spread following the early on addition of Pb^{2+} indicates that these events, in bone cells, are temporally mediated. Also, once spreading has developed Pb^{2+} cannot reverse this process. However, in our studies, no effect on cell attachment was noted in distinction to effects observed with marrow cells (Kowolenko *et al.*, 1989). Furthermore, cells already attached through a calcium dependent integrin receptor–fibronectin interaction were not detached by doses of Pb^{2+} that were sufficient to alter protein production and cell proliferation. The separate modulation of cell spreading and proliferation has been noted previously. Funk and Sage (1991) showed that SPARC inhibited cell proliferation and caused cell rounding of endothelial cells. Furthermore, inhibition of cell doubling was attributed to a 20-residue synthetic peptide (2.1) derived from a non-Ca^{2+}-binding, disulfide-rich region of SPARC, while cell rounding was promoted by sequences 5–23 in the calcium-binding region of the molecule. In the present study, there was no secretion of osteonectin/SPARC to cause cell rounding in the first few hours. However, Pb^{2+} might alter a membrane receptor, ion pump, or disturb a second messenger system (Borke *et al.*, 1988; Busselberg *et al.*, 1991) controlling cell shape.

Lead's ability to modulate both the synthesis and production of proteins by ROS 17/2.8 cells appears to be directed at least in part to acidic calcium binding proteins. Thus,

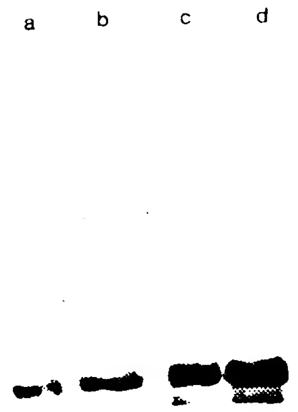

FIG. 4. Western blot of cellular osteonectin/SPARC (43 kDa) using anti-osteonectin antibodies, in ROS 17/2.8 cells following exposure to various concentrations of lead glutamate for 4 hr. (a) control; (b) 4.5×10^{-7} M Pb^{2+}; (c) 4.5×10^{-6} M Pb^{2+}; (d) 4.5×10^{-5} M Pb^{2+}. Lower molecular weight bands observed in lanes c and d were considered to represent minor alterations in protein processing, i.e., glycosylation or phosphorylation. Results represent the mean of three separate experiments.

FIG. 5. Immunoprecipitation of ^{35}S-methionine-labeled (4 hr) osteonectin/SPARC (43 kDa) from the media of ROS 17/2.8 cells following 4 hr treatment with 4.5×10^{-7} M Pb^{2+} and continuously labeled with ^{35}S-methionine. (a) control; (b) 4.5×10^{-7} M Pb^{2+}; (c) 4.5×10^{-6} M Pb^{2+}. Results represent the mean of three separate experiments.

collagen and Hsp47 levels are unchanged whereas cellular levels of osteonectin/SPARC are enhanced and secretion similar to osteocalcin (Angle *et al.*, 1990; Long *et al.*, 1989, 1990) is inhibited. However, the increased cellular levels of osteonectin/SPARC ensue while synthesis, depicted by short-term ^{35}S-methionine labeling, is diminished. Shelton *et al.* (1986) similarly demonstrated that Pb^{2+} enhances the level of an acidic (pI 6.3) inclusion body protein in kidney cells, and Fowler *et al.* (1985) showed increases in the synthesis of acidic gene products in rats given a single low dose of Pb^{2+} which induced intranuclear inclusions in renal proximal tubule cells. However, unlike the Pb^{2+}-induced nuclear proteins, osteonectin/SPARC is secreted, though delayed, in the presence of Pb^{2+} and is found in the medium. Lead inhibits this release possibly by affecting the conformation of osteonectin/SPARC as well as other acidic calcium-binding proteins. As a result, these proteins are retained in the ER. The inhibition or delayed secretion of structurally altered proteins is common and delayed synthesis, even in the presence of equivalent levels of mRNA, may ensue as a consequence of retention or delayed secretion of protein from the ER (Byers, 1990).

The diminished levels of osteonectin/SPARC mRNA in cells treated with Pb^{2+} for 24 hr signifies that pretranslational events controlling these acidic cellular proteins may also be affected by Pb^{2+}. The accumulation, over time, of Pb^{2+} in the nucleus (Shelton, 1986) may place Pb^{2+} in proximity to transcriptional events. Proteins such as p21/6.2, associated with nuclear matrices, have been implied in affecting DNA replication, RNA transcription, mitogenesis, and cell proliferation (Mistry *et al.*, 1985, 1986; Klann and Shelton, 1989; Fowler and Duval, 1991). Candidates for posttranscription and/or pretranslational affects of Pb^{2+} include the p220 component of an eukaryotic initiation factor 4F and regucalcin-like proteins. The p220 component functions in the first step of the binding of capped mRNAs to the small ribosomal subunit and has been shown to be a substrate for

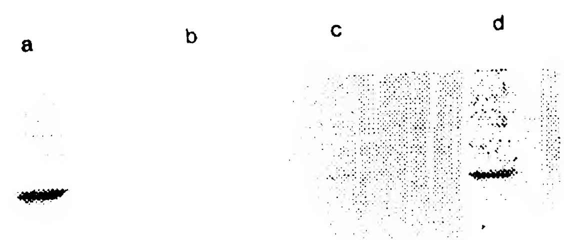

FIG. 6. Immunoprecipitation of cellular osteonectin/SPARC following 20 min treatment with various concentrations of lead glutamate. (a) control; (b) 4.5×10^{-5} M Pb^{2+}; (c) 4.5×10^{-6} M Pb^{2+}; (d) 4.5×10^{-7} M Pb^{2+}. Results were reproduced in [illegible] separate experiments.

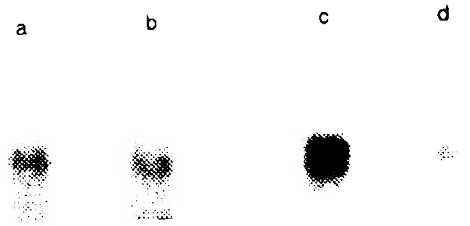

FIG. 7. Northern analysis of ROS 17/2.8 cells. Panel represents an autoradiogram of ^{32}P-nick-labeled bovine osteonectin cDNA hybridized to ROS 17/2.8 RNA migrating to 2.2 kb. (a) control cells, (b) 4.5×10^{-6} M Pb^{2+} for 1 hr, (c) control cells (24 hr), (d) 4.5×10^{-6} M Pb^{2+} (24 hr). Although equal amounts of RNA were loaded in each paring, autoradiograms of lanes c and d were exposed for a longer period in order to visualize lane d. Results were reproduced in two separate experiments.

multiple calcium-dependent enzymes (Wyckoff *et al.*, 1990). Regucalcin, a calcium-binding protein derived from cytosol, regulates Ca^{+2} mediated cell function by inhibiting aminoacyl-tRNA synthetase activity (Yamaguchi and Mori, 1990).

Thus, these studies demonstrate further that Pb^{2+} has a pleiotropic effect on bone cells. ER processes, possibly associated with calcium-dependent protein folding, appear to be sensitive to Pb^{+2}, resulting in retention or delayed protein secretion. In contrast, receptor-mediated attachment to substratum is undisturbed by Pb^{2+}. Future studies directed at establishing the mechanism for the alteration of specific bone proteins in cells exposed to Pb^{2+} are warranted and will help to clarify the effect of Pb^{2+} on bone growth and possibly provide a molecular marker for Pb^{2+} exposure in the environment.

ACKNOWLEDGMENTS

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thanks,
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THE DIAGNOSTIC VALUE OF IMMUNOHISTOCHEMICAL DETECTION OF OSTEONECTIN IN DIFFERENT TYPES OF OSTEOSARCOMA

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Summary

The pathologic diagnosis of osteosarcoma may be problematic due to wide histopathologic variations that can be mimicked by other primary or metastatic bone tumors with significantly different biologic potentials and treatment protocols. The accurate diagnosis of osteosarcoma relies on identification of malignant osteoblasts that are capable of producing neoplastic osteoid or bone matrix. To determine the benefit of immunohistochemistry for the diagnosis of osteosarcoma, twenty five cases of various types of osteosarcoma, seven fine needle aspirate of osteoblastic osteosarcoma, and eleven control lesions of nonosteogenic bone tumors were immunostained with monoclonal antibodies for osteonectin (a 32 Kda non collagenous bone protein). The production of osteonectin depends on the osteoblast-like function of the individual tumor cells, therefore, a homogenous immunocytochemical staining of all tumor cells and the surrounding matrix cannot be expected. Nevertheless, all cases of osteosarcoma demonstrated positive cell cytoplasmic and matrix staining with variable intensities. All control cases - except one case of conventional chondrosarcoma - showed negative cell cytoplasmic and matrix staining. In conclusion, immunohistochemical demonstration of osteonectin in osteosarcomas is a valuable tool for establishment of their osteogenic origin in order to differentiate them from other nonosteogenic bone tumors. The technique is reproducible in fine needle aspirates of osteosarcoma, which is considered as a noninvasive and easy diagnostic procedure.

Introduction

Osteosarcoma (OS) is the most common primary aggressive malignant bone tumor (1). Diagnosis of bone tumors is still based largely on the predominant matrix formation and the presumed cell of origin. Pattern recognition on conventional histologic sections is also a primary means of diagnosis (2). To qualify as an OS, a neoplasm should have proliferating malignant cells that produce either osteoid or bone at least in small foci. Thus, The presence of tumor osteoid or bone is essential for the diagnosis of OS (3-5).

Most OSs are easily diagnosed by light microscopy. The diagnosis however, may be problematic due to wide histopathologic

variations that can be mimicked by other primary and metastatic bone tumors (1). Moreover, small biopsy specimens may fail to show neoplastic bone or osteoid, necessary for light microscopic diagnosis. Other sarcomas may produce cartilage or hyalinized collagen that are difficult to distinguish from neoplastic osteoid (6,7).

Unfortunately, the accurate diagnosis of a material as osteoid is one of the major problems among bone pathologists, since there is no single histologic method that clearly identifies osteoid from hyalinized collagen (8). The same difficulties encountered by the light microscopy were faced with electron microscopy. (9). Ghadially and Mehta found that the fundamental units comprising the matrix of bone, cartilage, or fibrous tissue as well as their malignant tumors, are essentially similar since they show collagen fibers and fibrils set in mucopolysaccharide matrix (10). It was found that the most important sin-qua-non feature of osteoid is its ability to become mineralized by hydroxy apatite crystals which are not always identified in these lesions (10).

Bone matrix consists of type I collagen in addition to four non-collagenous proteins (osteopontin, bone sialoprotein, osteocalcin and osteonectin) (11). Osteonectin (32Kda) is a bone specific phosphorylated glycoprotein. Because its concentration is supposedly 500 to 1000 times in bone than in other tissues; it is thought to be a good marker for osteogenic lesions (5). Osteonectin is formed by cells, which possess the morphologic phenotype of osteoblasts and is believed to be a functional differentiation marker of osteoblastic lineage (5,12). It is greatly involved in the early steps of osteoid maturation and mineralization (5,13) by selectively binding type I collagen and hydroxy apatite crystals and controlling the size and speed of crystal formation and deposition (11).

Therefore, on this structural basis, a reliable immunohistochemical marker that would identify tumor cells that are capable of producing neoplastic bone and\or that would help to distinguish neoplastic osteoid from cartilage and collagen would be especially helpful in the diagnosis of OS. Immunohistochemical techniques using antibodies directed against different types of collagen did not prove helpful in separating cartilage-forming, bone-forming, and fibroblastic tumors (14). Recently, isolation of antibodies against bone-specific, non-collagenous matrix proteins such as osteonectin became possible for practical use (15-18).

The present work was designed with the aim of studying the degree of accuracy and validity of osteonectin as a tumor marker for diagnosis of OS, that can distinguish cells of osteoblastic phenotype from other mesenchymal cells and also neoplastic osteoid from other types of matrix such as cartilage or hyalinized collagen.

Material and Methods:

The material of the present study included biopsy specimens from 25 cases

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of OS. Fifteen were collected from the files of Pathology Department, Faculty of Medicine, Alexandria University and ten were prospective. Clinical and radiological data of all cases were available. Fine needle aspirations (FNA) from 7 cases of OS were included among the prospective examinations. As control lesions, eleven nonosteogenic bone tumors were included: conventional chondrosarcoma (two cases), mesenchymal chondrosarcoma, periosteal chondrosarcoma, malignant fibrous histiocytoma of bone (MFH) (one for each), Ewing's sarcoma (two cases) and giant cell tumor of bone (four cases).

For light microscopic and immunohistochemical staining, the surgical specimens were fixed in 10% buffered formalin, decalcified in 50% formic acid and 5% citric acid (whenever needed), and embedded in paraffin. Sections of 5-um thickness were prepared for staining with hematoxylin and eosin stains and for immunohistochemical staining.

All lesions were examined radiologically prior to performing FNA. When soft tissue component was present, or when the cortex was completely destroyed, 23-25 guage fine- needle was used. In other lesions, a Wescott needle was utilized. Fine needle aspirates were fixed in 95% alcohol for both hematoxylin and eosin stain and immunohistochemical staining.

Monoclonal osteonectin antibody commercially available as IgG, clone OC1 was used (Hematologic Technologies Inc). Immunohistochemical staining was accomplished by the avidin-biotin-peroxidase complex method of Hsu et al (19). The deparaffinized slides were incubated overnight with the primary antibody in a dilution of 1:50 at 4 C. Sections of human callus tissue were included as positive controls.

The results of osteonectin immunostaining in the present study were graded for tumor cell cytoplasmic staining and matrix staining on four- tiered grading system suggested by Fanburg-Smith et al (6): 1+ = focal (<50%) weak; 2+ = focal strong; 3+ = diffuse (>50%) weak; 4+ = diffuse strong. Negative immunoreactivity was graded as 0. All scores greater than 0 were interpreted as positive results.

Results

Clinical analysis of patients:

The material of the present study included 25 patients of osteosarcoma whose ages ranged from 11 to 36 years old (mean = 14.3). Sixteen patients were males with a sex ratio of 1.8:1. All patients were presented with pain (1.5-6 months). Eighteen patients complained of mass, and one was presented with pathological fracture.

The affected bones included the metaphysis of lower femur (nine; 36%), upper tibia (five; 20%), upper humerus (three; 12%), upper femur, upper fibula, and lower radius (two for each; 8%), midshaft femur and mandible (one for each; 4%).

Imaging findings:

Radiological examination of conventional OS revealed destructive

tumors arising from the medullary cavity of long bones and invading the cortex and surrounding soft tissues. Eleven lesions were osteoblastic and 13 were mixed osteolytic/osteoblastic with sunray pattern of new bone formation. (Fig1:1,2). One case of periosteal OS showed saucer-shaped osteolytic lesion in the outer cortex of upper tibia with intact medullary cavity.

Histopathological and immunohistochemical results:

The present study included 25 cases of OS that were subclassified into 23 conventional intramedullary OS, one low grade well differentiated intraosseous OS, and one periosteal OS. Conventional intramedullary OSs were subdivided according to the predominant pattern into 12 osteoblastic OS, three fibroblastic OS, three chondroblastic OS, two malignant fibrous histiocytoma subtype of OS, and one for each of giant cell rich OS, spindle cell type of small cell OS and telangiectatic OS.

Antiosteonectin antibody was immunoreactive in all cases of OS (100%). All variants of OS demonstrated positive cell-cytoplasmic and matrix staining with variable intensities.

Twelve cases of *osteoblastic OS* were encountered in the present study (fig1:3,4). Immunostaining of *osteoblastic OS* for osteonectin revealed intense positive cytoplasmic staining of malignant osteoblasts (4+). Areas of newly formed osteoid at osteoid seames, lace like osteoid and osteoid at the mineralization front showed intense positive staining (4+), whereas areas of old osteoid showed weak diffuse staining (3+). Mineralized osteoid and bone were negative (0). (fig 2:1-4)

FNA from seven cases of *osteoblastic OS* revealed highly cellular smear formed of pleomorphic population of cells with focal faint eosinophilic osteoid in between (fig1:5,6). Immunostaining for osteonectin revealed diffuse weak staining in different types of cells including polyhedral, spindle shaped, and mononucleated and multinucleated tumor giant cells (3+). Intense positive staining of osteoid matrix was also evident (4+) (fig2:5,6).

Immunostaining of three cases of *fibroblastic OS* for osteonectin revealed diffuse strong cytoplasmic staining of tumor cells (4+), and negative staining of intervening collagen fibers (0). Some fields of the tumor showed weak diffuse cytoplasmic positivity (3+). Positive staining of discrete foci of osteoid matrix (2+) was also observed (fig3:1,2).

In the same time, immunostaining of three cases of *chondroblastic OS* revealed diffuse weak cytoplasmic positivity of chondroblastic cells (3+) and negativity of chondroid matrix (0). Other few osteoblastic fields revealed positive cytoplasmic and matrix staining (2+) (fig3:3,4).

The histopathologic features of *MFH subtype of OS* were similar to those of pleomorphic MFH. Immunostaining for osteonectin revealed diffuse strong cytoplasmic positivity in fibroblastic spindle cells (3+), histiocyte-like polyhedral cells (4+), bizarre mononucleated and multinucleated tumor

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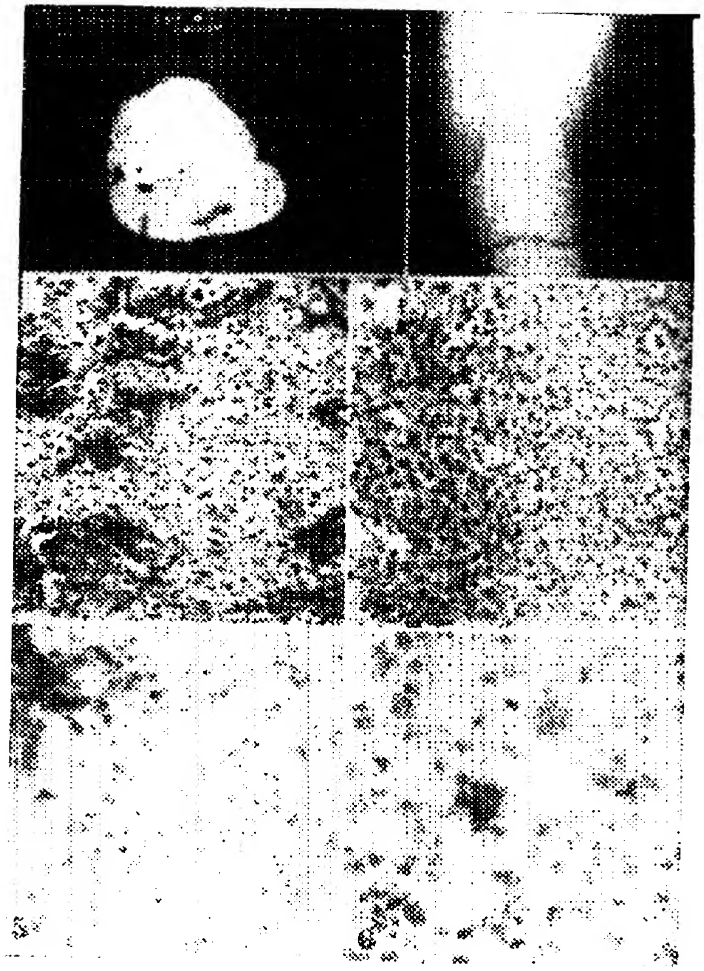

Fig 1

- CT scanning of osteoblastic OS of the tibia showing circumferential soft tissue invasion by the tumor tissue.
- Radiograph of osteoblastic OS of lower end femur showing sun burst pattern of new bone formation
- Osteoblastic OS showing spindle shaped to polyhedral tumor cells with hyperchromatic atypical nuclei and intervening wide seams of osteoid with focal mineralization. (H&Ex200)
- Osteoblastic OS showing lace-like osteoid. (H&Ex300)
- FNA of OS showing pleomorphic population of cells. (H&E [illegible])
- HPV of FNA of OS showing polyhedral, spindle shaped cells, and mononucleated and multinucleated tumor giant cells. (H&E [illegible])

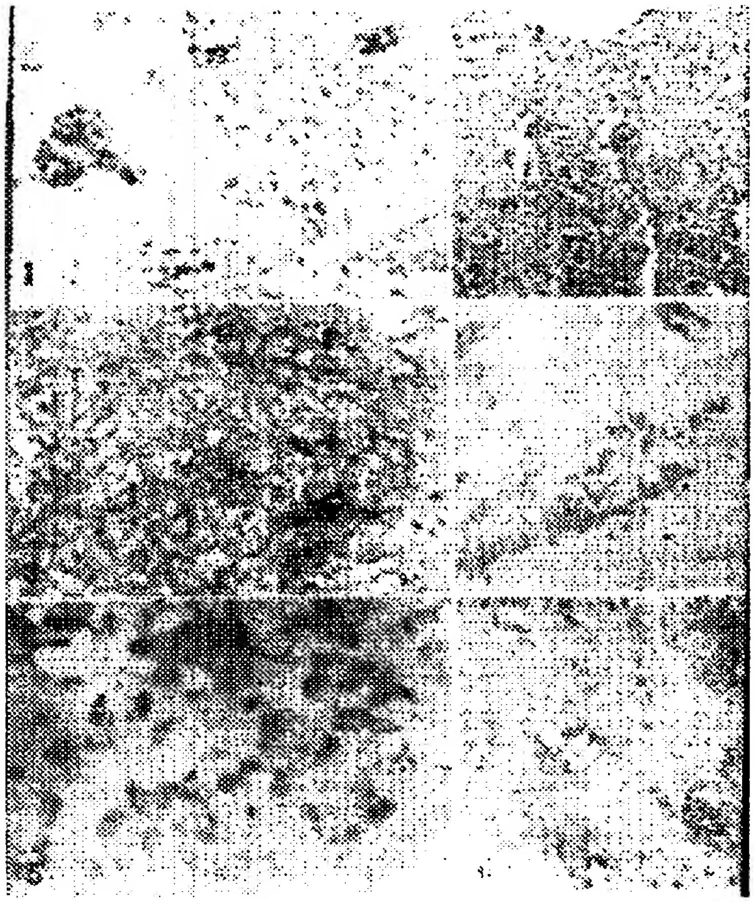

Fig. 2 Osteoblastic Osteosarcoma

1- Intense osteonectin immunostaining in tumor cell cytoplasm and osteoid matrix. (immunostain x 300)

2- Intense osteonectin immunostaining in tumor cells and lace-like osteoid (immunostain x 300)

3- HPV showing osteonectin positive lace-like osteoid and negative mineralized matrix (immunostain x 300)

4- Intense osteonectin immunostaining in osteoblasts and osteoid at the mineralization front and diffuse weak staining of old osteoid seams. (immunostain x 300)

5- FNA showing osteonectin immunostaining in spindle and polyhedral osteoblastic tumor cells as well as tumor giant cells (immunostain x 500)

6-FNA showing intense osteonectin immunostaining in osteoid matrix. (immunostain x 100)

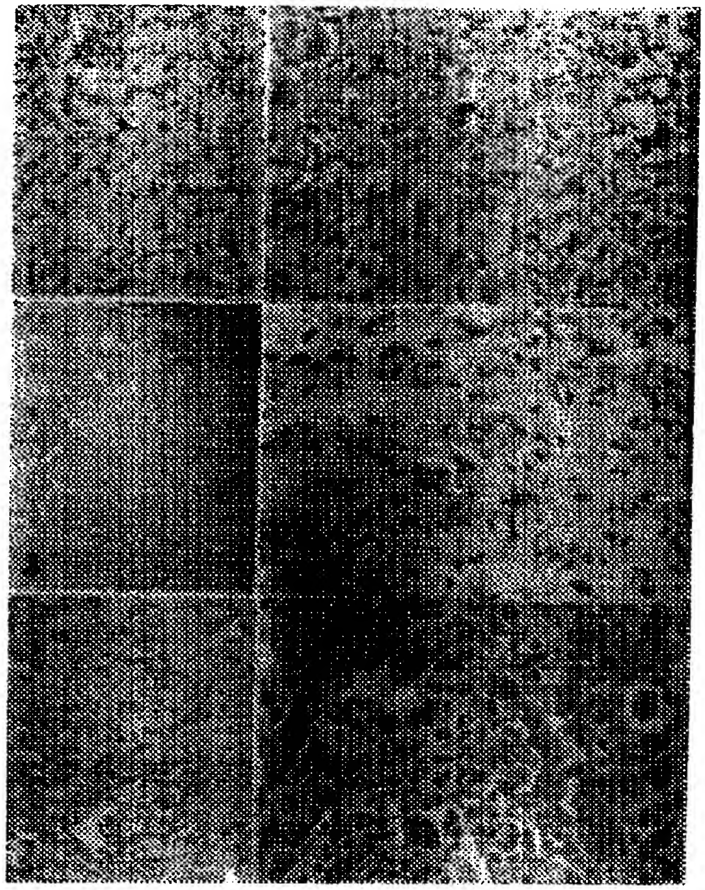

Fig 3

- fibroblastic OS showing spindle cells arranged in herring bone pattern with intervening fibrillary collagen matrix and focal osteoid. (H&E x300)
- fibroblastic OS showing intense osteonectin immunostaining in tumor cells and negative collagen fibers. (immunostain x 300)
- chondroblastic OS showing chondroid differentiation with focal osteoid. (H&Ex 300)
- chondroblastic OS showing weak diffuse osteonectin immunostaining in tumor cells and negative chondroid matrix.(immunostain x 500)
- MFH subtype of OS showing pleomorphic population of cells arranged in storiform pattern (H&Ex200)
- MFH of subtype of OS showing osteonectin immunostaining in spindle cells, polyhedral cells, bizarre tumor giant cells and osteoclastic giant cells. (immunostain x 500)

giant cells, and totoun giant cells (4+). Weak focal osteoid matrix positivity was observed (1+). (fig 3:5,6)

Immunostaining of one case of *giant cell rich OS* for osteonectin revealed strong positive cytoplasmic staining of osteoblastic malignant cells (4+). Osteoclastic giant cells were totally negative (0). (fig4:1,2)

Histopathologic examination of one case of *small cell OS* revealed a cellular tumor formed of closely backed spindle shaped cells displaying little visible cytoplasm with distinct cell borders. The nuclei were generally oval and small, but size did vary moderately. Few areas of narrow osteoid formation with focal mineralization surrounded by the spindle cells were found. Immunostaining for osteonectin revealed intense positive cytoplasmic staining of small spindle osteoblasts (4+) and the little tumor osteoid (4+). (fig4:3,4)

One case of *telagiectatic OS* revealed multiple vascular spaces surrounded by osteoblastic tumor cells. Immunostaining for osteonectin revealed diffuse intense cytoplasmic positivity of the spindle shaped tumor cells (4+). Strong positive staining of intervening osteoid matrix was detected (4+) (fig4:5,6).

Histopathologic examination of single case of *low- grade well-differentiated intra-osseous OS* revealed tumor tissue formed of spindle shaped cells with slightly atypical nuclei and collagen fibers in between. It showed multiple osteoid trabeculae with central mineralization surrounded by tumor cells. Immunostaining for osteonectin revealed diffuse weak cytoplasmic staining (3+) as well as weak focal positive staining of osteoid matrix (1+), whereas negative staining of mineralized matrix and collagen fibers was evident (0) (fig5:1,2).

Periosteal OS was encountered in the present study in one case. The tumor was formed of malignant chondroid lobules arranged in parallel fashion perpendicular to the cortex. The lobules were separated by spindle cells. Fine lace-like osteoid was present in the central portion of the lobules giving a feathery appearance, as well as in between the peripheral spindle cells. Immunohistochemical staining of osteonectin revealed positive cytoplasmic staining of spindle shaped cells at the periphery of the lobules (4+) and malignant chondroblasts at the central portion (+3). Fine lace-like osteoid at the center and periphery of the lobules was intensely positive (4+) but the chondroid matrix was negative (0)(fig5:3-5).

Immunostaining of control cases of conventional chondrosarcoma for osteonectin revealed weak focal cytoplasmic reactivity only in malignant chondrocytes in the vicinity of mineralized central zone (1+) in one out of two cases (fig5:6). Lacunar chondrocytes at the periphery of the lobules as well as spindle shaped cells were totally negative. No matrix staining was observed anywhere in the tumors (0).

The other nine control tumors including mesenchymal chondrosarcoma, periosteal chondrosarcoma, giant cell tumor, MFH, and Ewing's sarcoma showed total cytoplasmic and matrix negativity (0).

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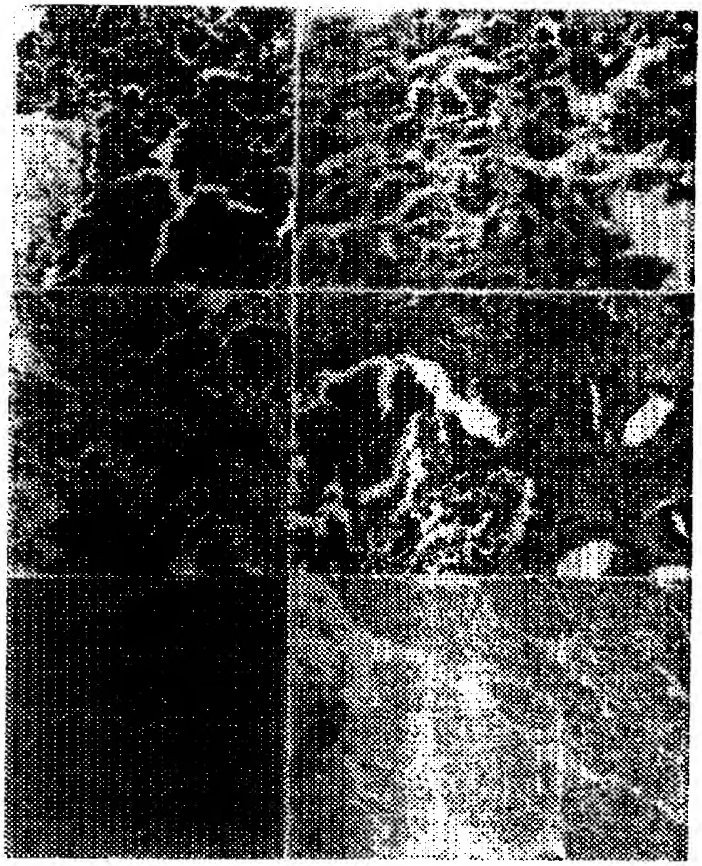

Fig 4
- Giant cell rich OS showing many osteoclastic giant cells scattered between spindle shaped osteoblasts. (H&Ex200)
- Giant cell rich OS showing intense osteonectin immunostain in osteoblastic tumor cells and negative osteoclastic giant cells (immunostain x400)
- Small cell OS showing small spindle shaped cells (H&Ex300)
- Small cell OS showing intense osteonectin immunostaining in tumor cells and scanty osteoid matrix. (immunostain x200)
- Telangiectatic OS showing many vascular spaces lined by the tumor cells. (H&Ex 100)
- Telangiectatic OS showing intense osteonectin immunostaining in tumor cells and intervening osteoid matrix. (immunostain x200)

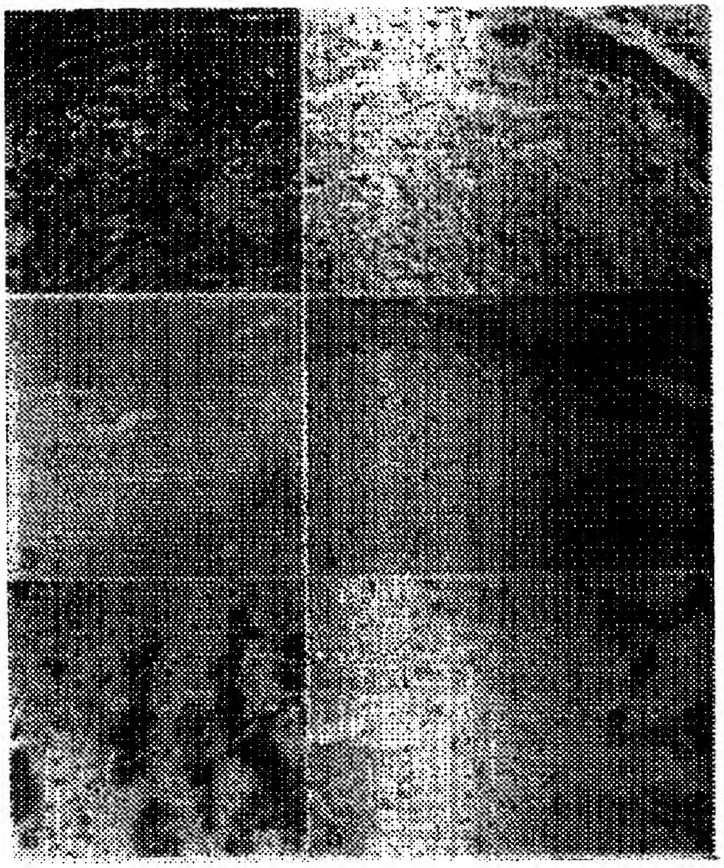

Fig 5

- Well differentiated intraosseous OS showing spindle shaped cells and intervening collagen fibres and osteoid trabecula with central mineralization (H&Ex200)
- Well differentiated intraosseous OS showing osteonectin immunostain in tumor cells and osteoid trabecula, but negative staining of collagen fibers. (immunostain x200)
- Periosteal OS showing lobules of chondroid differentiation with central and peripheral osteoid. (H&Ex80)
- Periosteal OS showing osteonectin immunostaining in peripheral spindle cells and intervening osteoid, but negative chondroid matrix. (immunostain x200)
- Same case showing intense osteonectin immunostaining in central lace-like osteoid (immunostain x100)
- Conventional chondrosarcoma showing osteonectin immunostaining in tumor cells only at the vicinity of mineralization (immunostain x200)

Discussion

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Discussion

The constellation of different histopathologic patterns of OS, not only among tumors but also from one microscopic field to the next, has been emphasized and delineated by Yunis and Barnes, allowing wide subclassification of OS (20). In addition to histopathological variations, these subtypes show behavioral and radiological differences despite the fact that they all share the appellation of OS. The critical importance of this subclassification is that one must be aware of, and become familiar with the histopathologic variants if major diagnostic errors are to be avoided.

OSs produce tumor osteoid as extracellular matrix (ECM) which comprises the microscopic hallmark of these tumors. It can be difficult to differentiate tumor osteoid from other ECMs produced by intra- or extraskeletal tumors, so the problem of differential diagnosis arises. Conventional and special stains provide a mean to increase the reliability of the differential diagnosis, but do not identify the type of tumor conclusively as they only reflect physiochemical features and do not identify the molecular components of the tumor matrix. The key solution of this clinicopathologic problem is the immunohistochemical use of antibodies against bone matrix components such as osteonectin (21).

Osteonectin is an adhesive matrix glycoprotein, which is present in active osteoblasts and osteoprogenitor cells as well as in young osteocytes but not in quiescent inactive osteocytes (16). The intracellular distribution of osteonectin in active osteoblasts is found as a faint immunolabeled substance in vesicular Golgi fields and some lamellae of rough endoplasmic reticulum. Intensive labeling occurs in opaque cytoplasmic vesicles pointing to the extracellular secretion as some of the vesicles are connected with the basal cell membrane. Extracellularly, osteonectin binds first to the deeper layer of osteoid maturation with intensity increasing at the mineralization front (13). The role of osteonectin in mineralization and osteoblastic differentiation is believed to be affected by various growth factors and cytokines (22).

Since the production of osteonectin depends on the functional status of osteoblasts (16), no homogenous immunohistochemical staining of all tumor cells and surrounding matrix should be expected. It was therefore necessary to examine various types of OS as regards the ability of tumor cells to produce osteonectin.

In the present study osteonectin antibody was immunoreactive in 100% of collected cases of OS. Osteonectin was expressed at least focally in the cytoplasm of malignant osteoblastic cells in all OS variants. Immunoreactivity of osteoid matrix for osteonectin was positive in all cases with variable intensities at variable fields. The maximal intensity was observed in the deeper layer of osteoid maturation at the mineralization front which is similar to the reported pattern of immunostaining of osteonectin studied in normal osteoblastic

bone matrix (13). The overall sensitivity of osteonectin antibody was 100% when tumor cell cytoplasmic staining and matrix staining were evaluated together. This result is similar to that reported by other authors (5,6,17,23). In the present study, 100% of FNA of OS revealed positive cell cytoplasmic and matrix immunoreactivity, which emphasizes the importance of osteonectin antibody in the diagnosis of OS by this semi- invasive and easy technique. To my knowledge this is the first study dealing with osteonectin immunoreactivity in FNA of osteosarcoma.

In the present study, one control case of conventional chondrosarcoma revealed focal faint cytoplasmic staining in chondrocytes surrounded by mineralized bone matrix. A similar finding was reported by Jundt et al who emphasized that only chondrocytes surrounded by mineralizing matrix containing type I collagen showed a positive reaction to osteonectin antibody (12). Chiba et al reported a similar observation explaining that osteonectin plays an important role in calcification of normal and neoplastic cartilage tissue (24). Osteonectin immunoreactivity for other ten control cases of nonosteogenic tumors revealed negative staining. This finding was similarly reported by other authors who asserted negative staining for osteonectin in conventional and mesenchymal chondrosarcoma, Ewing's sarcoma, fibrosarcoma, and MFH (12,17,25-27). These findings intensify the value of osteonectin antibody in the differential diagnosis of osteosarcoma from non- osteogenic bone tumors.

Oppositely, other authors reported different results showing positive staining for osteonectin in nonosteogenic tumors including non bone-forming tumors, such as, chondrosarcoma, giant cell tumor, MFH, and Ewing's sarcoma, as well as melanomas, carcinomas, and lymphomas (6,18,28-30). Metsaranta et al attributed these results to the effective presence of an osteoblastic component within the tumor cells or to the re-establishment of fetal functional characteristics in the neoplastic phenotype (31). Serra et al related them to differences in the antibody concentration and in the immunohistochemical methods (25). Therefore, authors concluded that osteonectin antibody is not specific for OS cells as regards the cytoplasmic positivity. However, they found that chondroid matrix and extracellular collagen, which may mimic osteoid when examined by the light microscopy, did not react with osteonectin antibody in any of the tumors. Similarly, they reported that osteonectin antibody helps to distinguish neoplastic osteoid, which is essential for the diagnosis of OS (6,18,25,28-30).

In the present study, fibroblastic OS revealed strong diffuse cytoplasmic immunoreactivity to osteonectin. Fields of focal weak cytoplasmic staining may be explained by the postulation of Ferguson and Yunis that spindle shaped cells in areas of fibroblastic differentiation of OS may represent nonfunctioning or resting osteoblasts that have lost the organelles essential for matrical protein synthesis and have assumed the spindle shape characteristic of fibroblasts (1).

Among the various difficult bone tumors to diagnose, MFH-subtype of OS remains the most confusing entity. It is virtually indistinguishable from malignant fibrous histiocytoma (MFH) of bone or soft tissues, as a result of which, diagnostic errors of considerable therapeutic significance can ensue. Although both are high-grade sarcomas, therapeutic modalities, especially with respect to chemotherapeutic protocols, may differ (32).

Authors had clearly distinguished primary MFH of bone from MFH subtype of OS by the presence of neoplastic osteoid or woven bone in the latter and their absence in the former (32). This neoplastic osteoid must be differentiated from reactive osteoid which shows prominent monolayer of benign osteoblasts rimming it. Authors also reported that highly malignant cells of OS may develop the properties of histiocytes. That is to say, a malignant tumor in which histiocyte- like cells produce osteoid or bone is an OS of the MFH subtype (33). In the present study, this finding was documented not only by the use of conventional histologic techniques, but also by accurate immunohistochemical detection of osteoid and osteoblasts using osteonectin antibody. Balance et al had emphasized that the extraosseous component of MFH subtype of OS is devoid of osteoid or bone, and thus could not be identified form MFH of bone or soft tissues (34). The osseous component, however, contains an abundant of tumor osteoid and woven bone in all cases, a finding which was explained by Yoshikawa et al by the fact that bone milieu- possibly bone morphogenetic proteins - may be influential in the genomic programming of selective tumor cells to synthesize osteoid (35). This finding emphasizes the difficulties that may be present in a limited biopsy specimen, or fine needle aspirate from the soft tissue component of MFH subtype of OS, as well as the importance of using osteonectin as a tumor marker for solving such diagnostic problem by detection of minimal amounts of osteoid and identification of osteoblasts (34).

Giant cell rich OS is a subgroup of OS that is difficult to differentiate from giant cell rich malignant fibrous histiocytoma (6). In the present study the positive immunoreactivity of tumor cells for osteonectin antibody helped to confirm the osteoblastic nature of these cells. Osteoclastic giant cells showed total negativity for osteonectin antibody. Fanburg-Smith et al. however, reported nonspecific staining of osteoclastic giant cells in these tumors and in giant cell tumor of bone (6). Hasegawa et al reported similar finding and referred this to the phagocytosis of bone matrix by osteoplasts (36).

Small cell OS is another distinct clinicopathologic entity within the class of high-grade OS. Sim et al believed that the prognosis could be worse than the prognosis for conventional OS (37). The histopathologic features of small cell OS of the present study were similar to that reported by many authors (37-39). It may be critical to the diagnosis if the biopsy material does not clearly include

osteoid matrix (40,41). In such a condition, the differential diagnosis of spindle cell type of small cell OS includes the spindle cell pattern seen in monophasic synovial sarcomas, and mesenchymal chondrosarcoma. Both tumors, however, lack osteoid formation by the malignant cells (41). Immunohistochemical staining using osteonectin antibody could be of help by verifying the histogenetic type of these spindle cells as osteoblasts. The focal lower yield of cytoplasmic reactivity to antiosteonectin antibodies suggests that these cells produce non-collagenous bone proteins in undetectable amounts, or perhaps they do not produce them at all. Similar findings for immunoreactivity of small cell OS have been reported in the literature (6,25,40,41).

Low-grade, well-differentiated intraosseous OS is a rare subtype. It is sometimes difficult to recognize it as a low-grade malignant lesion. It has a better prognosis so that it is worthwhile to differentiate it from the more malignant varieties of intraosseous OS. Differentiation from other benign intraosseous lesions with which it can be confused easily, especially fibrous dysplasia, is mainly histologic and depends on identification of nuclear atypia, hypercellularity and bone marrow and periosteal invasion which are constant features of low grade osteosarcoma and are never observed in fibrous dysplasia (42-45).

Periosteal OS, which is a distinct entity, is an extremely rare bone tumor (1). The prognosis of periosteal OS is much better than that of conventional intramedullary OS and high grade surface OS (46,47). The differential diagnosis could be a problem when the latter shows predominant chondroid differentiation. Similar to the finding of the present study, Okada et al reported that the most important diagnostic feature of periosteal OS is the perpendicular arrangement of chondroid lobules and fine lace-like osteoid, which is present most often in the center of the lobules or among peripheral spindle cells (47). In the present study, the positive immunoreactivity of the central lace-like osteoid matrix and the cytoplasm of the peripheral spindle-shaped osteoblasts confirmed the osteoblastic origin of the tumor and helped to differentiate it from periosteal chondrosarcoma that showed total negativity of chondroblastic cells and matrix. The same findings were reported by Franchi et al (23).

In conclusion, Osteonectin is a sensitive and specific marker for osteoblastic cells and osteoid matrix, which are essential for the diagnosis of OS, and therefore it would be extremely helpful in identifying them in small biopsy specimens and in fine needle aspirates. The pathologist must be cautious when interpreting small sections taken from tumor-soft tissue interface as well as from the periosteum since woven bone or osteoid at these locations may be reactive rather than neoplastic. Recent advances emphasized the role of osteonectin- rather than tissue mineralization- in cell adhesion, migration, differentiation, and proliferation, as well as in angiogenesis (48-51). More recently, authors assessed the circulating tumor

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burden in osteosarcoma patients by semiquantitative reverse transcriptase PCR using osteonectin mRNA as molecular marker, to monitor tumor dissemination and micrometastasis (52).

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Comparison of gene expression profiling between malignant and normal plasma cells with oligonucleotide arrays

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The DNA microarray technology enables the identification of the large number of genes involved in the complex deregulation of cell homeostasis taking place in cancer. Using Affymetrix microarrays, we have compared the gene expression profiles of highly purified malignant plasma cells from nine patients with multiple myeloma (MM) and eight myeloma cell lines to those of highly purified nonmalignant plasma cells (eight samples) obtained by *in vitro* differentiation of peripheral blood B cells. Two unsupervised clustering algorithms classified these 25 samples into two distinct clusters: a malignant plasma cell cluster and a normal plasma cell cluster. Two hundred and fifty genes were significantly up-regulated and 159 down-regulated in malignant plasma samples compared to normal plasma samples. For some of these genes, an overexpression or downregulation of the encoded protein was confirmed (cyclin D1, c-*myc*, BMI-1, cystatin c, SPARC, RB). Two genes overexpressed in myeloma cells (*ABL* and cystathionine beta synthase) code for enzymes that could be a therapeutic target with specific drugs. These data provide a new insight into the understanding of myeloma disease and prefigure that the development of DNA microarray could help to develop an 'à la carte' treatment in cancer disease.
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Keywords: myeloma; plasma cell; DNA microarray; c-*myc*; abl; cystathionine beta synthase

Introduction

Accumulating evidence has suggested that the transformation of a normal plasma cell into a malignant myeloma cell is a multiple-step process (Bergsagel and Kuehl, 2001; Hallek *et al.*, 1998). Genotypic changes are found in 60% of patients at diagnosis by conventional karyotyping and in up to 90% of patients by FISH analysis. Some of these genetic abnormalities have been identified. Overall there is a rearrangement of the *14q32* (IgH) locus in 60% of patients, c-*myc* in 15–50%, cyclin D1 in 20%, FGFR3 in 10%, cyclin D3 in 5%, as well as other genes not yet identified. In addition, 40% of multiple myeloma (MM) cases display monoallelic deletions of chromosome 13 in association with the loss of one retinoblastoma allele. Mutations of the K-ras and N-ras genes are found in 50% of the patients at diagnosis and p53 monoallelic loss in 20%. Phenotypic changes have also been described such as the loss of CD19 and aberrant expression of CD56. Moreover, the bone marrow environment is highly activated in MM, as evidenced by a high stimulation of neoangiogenesis, a production of myeloma cell growth factors such as IL-6 (Klein *et al.*, 1995), a production of IL-6 inducers such as IL-1 (Costes *et al.*, 1998), and stimulation of bone resorption. This abnormal hyperactivation of the bone marrow environment in patients with MM is the result of synergistic interactions between the malignant plasma cells and specific cells in the tumor environment, likely involving various pathways of intercellular communication.

The portrayal of this complex alteration of the cellular circuitry and cellular behavior in myeloma cells will best be apprehended by the use of DNA microarrays. Indeed, gene-expression profiling using microarrays allows the simultaneous analysis of multiple markers and is thus an ideal tool to study the global changes that drive a normal cell to malignancy (Alizadeh *et al.*, 2000; Thykjaer *et al.*, 2001). We recently used nylon cDNA macroarrays and identified cytokines and cytokine receptors overexpressed in human myeloma cell lines (HMCL) compared to their autologous, EBV-transformed B cell lines (De Vos *et al.*, 2001). The recent description by our group of a procedure allowing the generation of nonmalignant polyclonal plasma cells in patients with myeloma provides us with the opportunity to compare the gene expression profile of malignant plasma cells directly with their normal autologous counterpart (Tarte *et al.*, 2002). Oligonucleotide microarray analysis between

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myeloma cells, polyclonal plasma cells, and B cells can identify genes whose expression was restricted to plasma cells, i.e., plasma cell genes (Tarte *et al.*, 2002). This technique also opened the way to a molecular classification of this heterogeneous disease (Zhan *et al.*, 2002). In this study, we have analysed genes that are overexpressed or underexpressed in myeloma cells compared to normal plasma cells. Among the numerous genes that have a higher expression level in malignant plasma cells, we have focused our study on a few genes that encode for proteins that could be involved in myeloma biology or could be potential therapeutic targets.

Results and Discussion

Unsupervised clustering

The myeloma samples comprised highly-purified myeloma cells (>95%) from six patients with medullary MM (MM1-MM6), from three patients with plasma cell leukemia (PCL1–PCL3) (Table 1), and eight myeloma cell lines (XG-1, XG-5, XG-6, XG-7, XG-13, XG-14, XG-16 and RPMI8226). The eight normal plasma cell samples were *in vitro*-generated polyclonal plasmablastic cells (PPC1–PPC7) and highly purified plasmablasts (>99%) from one patient with reactional polyclonal plasmacytosis (RP). The gene expression profiling of the samples was determined with Affymetrix HuGeneFL arrays (6800 genes) and the Affymetrix files are available as supplementary data (http://www.u475.montp.inserm.fr/BK/SupplementalData.htm). Data were processed as indicated in Materials and methods and 3874 genes were retained for analysis. The hierarchical clustering method delineated two major clusters: one cluster comprising the *in vitro*-generated PPC and the RP sample that was termed the PPC cluster and a myeloma cluster (Figure 1a). Of note, the three PPCs obtained from myeloma patients (i.e., PPC4, PPC5, and PPC6) and from healthy donors (i.e., PPC1, PPC2, and PPC3) segregated together and were intermingled. The myeloma cluster was subsequently divided into two subclusters, a primary myeloma cell cluster comprising MM1–MM6 and PCL1 and PCL3 (subsequently called the MM cluster) and a myeloma cell line cluster, which comprised all HMCLs included in this study and primary myeloma cells from one patient with PCL PCL2 (termed the HMCL cluster). Interestingly, we have been able to grow PCL2 cells *in vitro* – thereby generating a new IL-6-dependent HMCL (XG-19) – but not PCL1 or PCL3 cells. Principal component analysis (PCA) was used as a second unsupervised method to classify samples. The first three principal components retained 46.6% of the total variance and allowed us to display our 25 samples in a space with three dimensions (Figure 1b). In complete agreement with the hierarchical clustering, the PCA method delineates three clusters of samples: PPC, MM (MM1–MM6, PCL1 and PCL3), and HMCL (HMCL and PCL2). Thus both average linkage clustering and PCA recognized the different biologically relevant sample groups, suggesting that the oligonucleotide arrays could indeed apprehend the molecular differences that distinguish normal plasma cells from myeloma cells.

Table 1 Patient characteristics

Patient	*Gender*	*Age* (y)	*Paraprotein*	*Stage*	*Diagnostic/Relapse*
MM1	M	64	IgGλ	IIIA	D
MM2	F	52	IgGκ	IIIA	D
MM3	M	43	IgAκ	IIIA	D
MM4	F	64	IgGκ	IIIA	D
MM5	M	66	IgGλ	IIIA	D
MM6	M	68	IgAλ	IIA	D
PCL1	F	52	λ	PCL	1st R
PCL2	F	50	IgAλ	PCL	D
PCL3	M	63	κ	PCL	D

Abbreviations: D, diagnostic; R, relapse

Comparison of gene expression between malignant and normal plasma cells

Gene expression between malignant and normal plasma cells was compared with the Mann–Whitney nonparametric statistical test. Out of the 3874 genes retained in the analysis, 250 were significantly ($P \leqslant 0.05$) overexpressed in malignant plasma cell samples compared to normal plasma cells, with a ratio of mean expression values $\geqslant 2$. Similarly, 159 genes were underexpressed in malignant plasma cell samples, with a ratio of mean expression values $\leqslant 0.5$. Tables 2 and 3 (shown as supplementary information, http://www.u475.montp.inserm.fr/BK/SupplementalData.htm) list these 409 genes that are significantly overexpressed or underexpressed in MM, respectively. In the following, we have focused on genes that validated our microarray data, could be of major importance for the emergence of the malignant plasma cell clone, or could be potential therapeutic targets. These genes and their expression are shown in Figure 2.

Altered expression of cell cycle genes in myeloma samples

Cyclin D1 Cyclin D1 is one of the most differentially expressed genes between normal and malignant plasma cells (Table 2). The up-regulation of this oncogene is in good agreement with the recurrent t(11;14)(q13;q32) translocation observed in myeloma cells, involving the immunoglobulin heavy (Ig H) chain gene and the cyclin D1 gene, whereas cyclin D1 is not expressed in normal hematopoiesis and lymphopoiesis (Bergsagel and Kuehl, 2001). The microarray results were validated by the known cytogenetics of our cell lines since a t(11;14)(q13;q32) translocation has been documented in the XG-1 and XG-5 HMCLs (Raynaud *et al.*, 1993), and a more complex chromosome 11 abnormality, der(11)t(1;3;11), was reported in XG-6 by spectral karyotyping (Rao *et al.*, 1998). The Affymetrix microarray data were also in good agreement with the WB analysis that demonstrated a strong *cyclin D1* expression in XG-1, a weak expression in XG-5 and

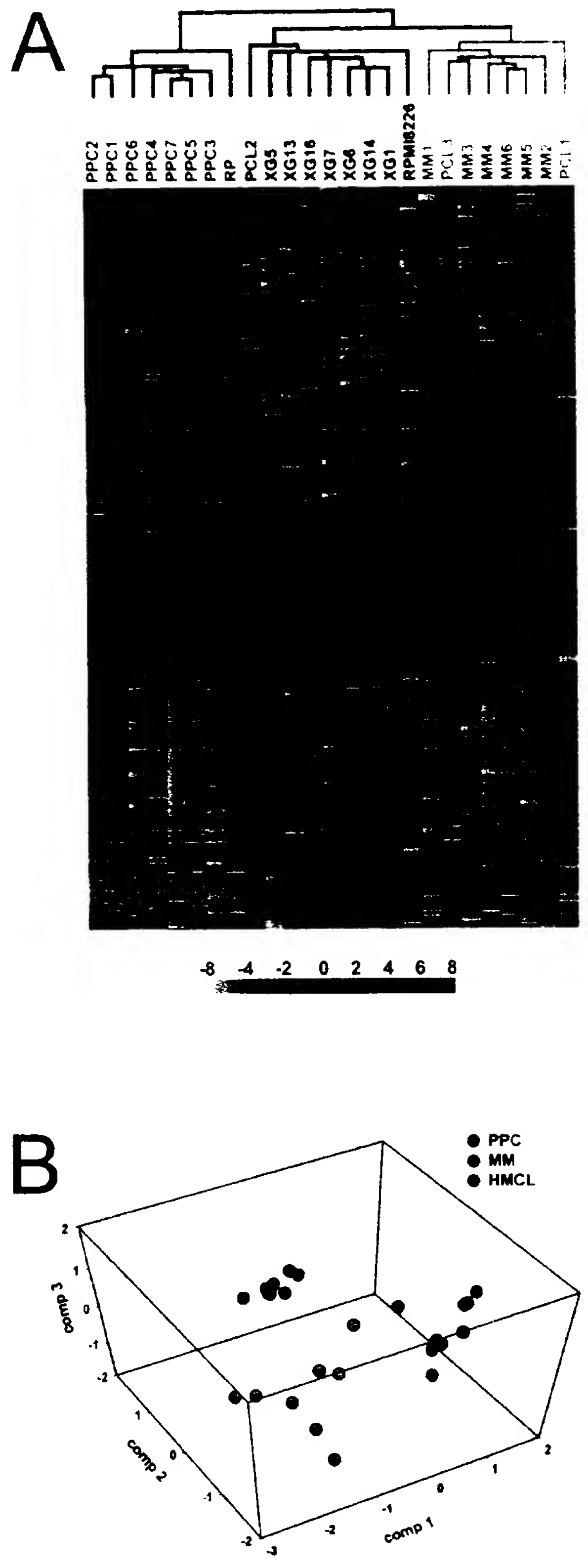

Figure 1 Unsupervised clustering of normal and malignant samples. (**a**) Hierarchical clustering of 3874 genes based on their expression in normal and malignant plasma cell samples. Each value represents the difference from the gene median and is depicted according to the color scale shown at the bottom (−8 to 8 on a log base 2 scale). The cluster dendrogram showing the three sample clusters is colored as: normal plasma cells, blue; primary malignant plasma cells, orange; HMCLs, red. RP, reactive plasmacytosis; PPC, polyclonal plasmablast cells; MM, primary medullar myeloma samples; PCL, primary plasma cell leukemia. (**b**) Principal component analysis (PCA) of the 1500 genes with the highest variation coefficient among the 25 samples. The axes represent the first, second, and third components that account for most of the variation across the data set. Sample color code is the same as in (**a**)

-14 HMCLs, and no expression in the other five cell lines (Figure 3a).

Myc and myc partners The Affymetrix microarrays showed a 7.5-fold overexpression of the c-*myc* gene in myeloma cells compared to normal plasma cells, which confirms the frequent overexpression of the c-*myc* oncogene in myeloma cells reported by others (Shou *et al.*, 2000). The low expression of the c-*myc* gene in normal plasma cells is in agreement with the reported silencing of this gene during normal plasma cell differentiation by PRDI-BF1. We have previously found that these *in vitro*-generated normal plasma cells expressed PDRI-BF1 (Tarte *et al.*, 2002). Using WB, we have confirmed that eight out of eight of the HMCLs overexpressed the c-*myc* protein, unlike normal plasma cells (Figure 3b). The very high c-*myc* gene and protein expression in RPMI8226 cells is in agreement with a reported c-*myc*-IgLλ rearrangement in this HMCL (Avet-Loiseau *et al.*, 2001). In addition, we found an overexpression in myeloma samples of three other genes, which either encode for proteins that cooperate with c-*myc* during oncogenesis (BMI-1 and MSSP, c-*myc* single-strand binding protein) or are essential to c-*myc* function (GCN5L2, general control of amino-acid synthesis-like 2). Regarding BMI-1, we have confirmed by WB a higher expression in five out of eight myeloma samples as compared to normal plasma cells (Figure 3c). BMI-1 is an oncogene that was first identified because of its cooperation with c-*myc* for lymphoid transformation *in vivo* (van Lohuizen *et al.*, 1991). A recent study has revealed that BMI-1 is a transcription repressor that targets the ink4a locus, which encodes the p16 protein and that BMI-1 cooperates with c-*myc* by repressing p16 and P19 ARF. Of note, the p16 protein is often absent in myeloma cells (Ng *et al.*, 1997). Though a promoter hypermethylation has been invoked, our data suggest that BMI-1 could contribute to p16 silencing in MM. The second *myc* partner gene upregulated in myeloma samples is MSSP. MSSP is a *myc*-mediated transcription modulator and stimulates the cooperative transforming activity of c-*myc* with ras. The general up-regulation of c-*myc* and the frequent activating ras mutation in myeloma could thus see their transforming properties enhanced in myeloma cells by the overexpression of MSSP. Finally, the third c-*myc* partner gene upregulated in myeloma samples codes for GCN5, a histone acetyltransferase which plays an essential role in the transcriptional activity of c-*myc*. Overall, our microarray data highlight a pivotal role for an up-

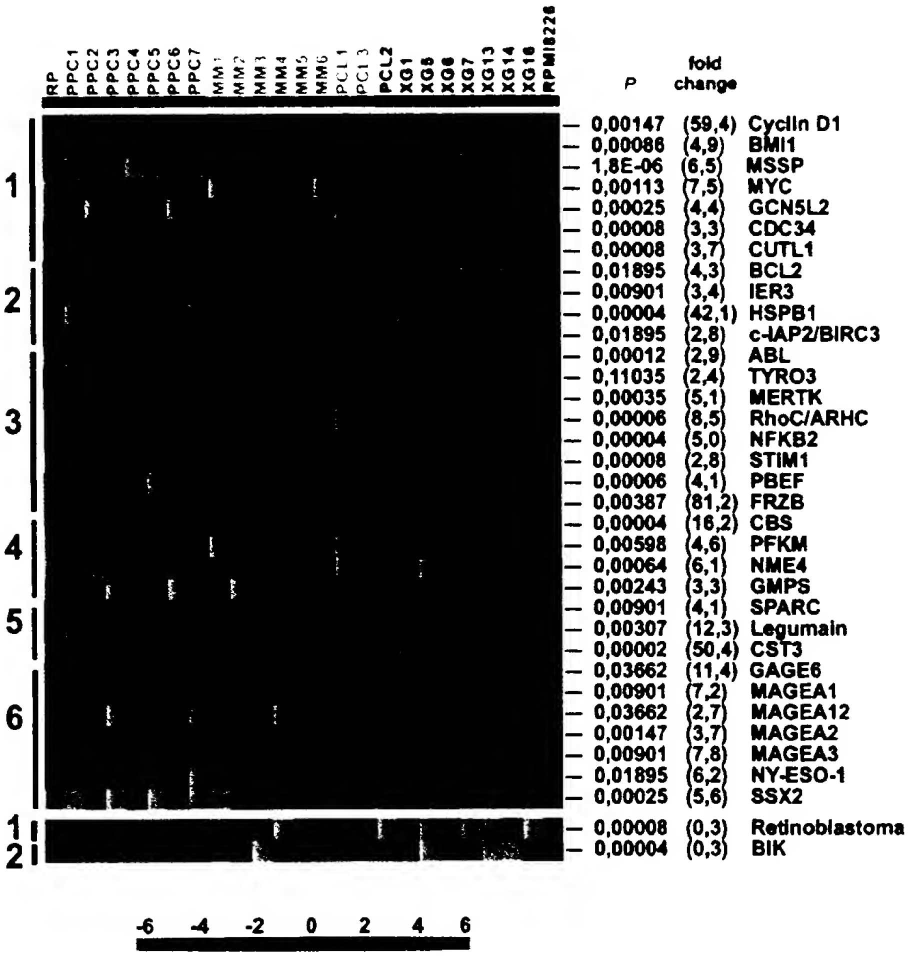

Figure 2 Genes differentially expressed between normal and malignant plasma cells. Visualization by Treeview of genes that are significantly (except Tyro 3) over- or under-expressed in malignant plasma cell samples compared to normal plasma cell samples and that are discussed in the text. Color code for samples is as in Figure 1. Each value represents the difference from the gene mean and is depicted according to the color scale shown at the bottom (-6 to 6 on a log base 2 scale). The *P*-value obtained by the Mann–Whitney test and the fold change is indicated for each gene. 1, proliferation; 2, apoptosis; 3, transduction; 4, metabolism; 5, bone remodeling; 6, cancer/testis antigens

regulation of the c-*myc* pathway in MM. It is noteworthy that a deregulation of the c-*myc* gene is an obligatory oncogenic event to create a malignant plasma cell tumor in mice (Potter and Wiener, 1992).

Other cell cycle genes We found that the CDC34 gene was up-regulated in 11 out of 14 MM samples. Of note, this gene has already been reported to be up-regulated in acute lymphoblastic leukemia and hepatocellular carcinomas (Tanaka *et al*., 2001). CDC34 is a ubiquitin-conjugating enzyme that appears to play a particularly important role in the control of the cell cycle in budding yeast and Xenopus (Michael and Newport, 1998). CDC34 is also probably involved in the regulation of the cell cycle of mammalian cells, given its high conservation in eukaryotic cells. Cut-like 1 (CUTL1), which has a demonstrated role as an inhibitor of the p21 cyclin kinase inhibitor (Coqueret *et al*., 1998), was also overexpressed in MM samples as compared to PPC. Finally, analysis of genes down-regulated in MM samples identifies the tumor suppressor retinoblastoma (Rb). This lower expression could be related to the high incidence of monoallelic deletion in MM cells (Juge-Morineau *et al*., 1995). WB confirmed the higher level of Rb protein in normal plasma cells compared to MM cells (Figure 3d). Thus, though we compared our MM samples to nonmalignant plasmablasts which still retain a high mitotic index (Tarte *et al*., 2002), in malignant plasma cells we found an altered expression pattern of several genes involved in cell cycle regulation.

Altered expression of apoptosis genes in myeloma samples

Gene alterations leading to increased cell survival are frequent in cancer. A striking difference between normal and malignant plasma cells is the up-regulation of the anti-apoptotic Bcl-2 gene and the down-regulation of the pro-apoptotic BIK (Bcl2-interacting killer) gene in MM samples. A high expression of Bcl-2

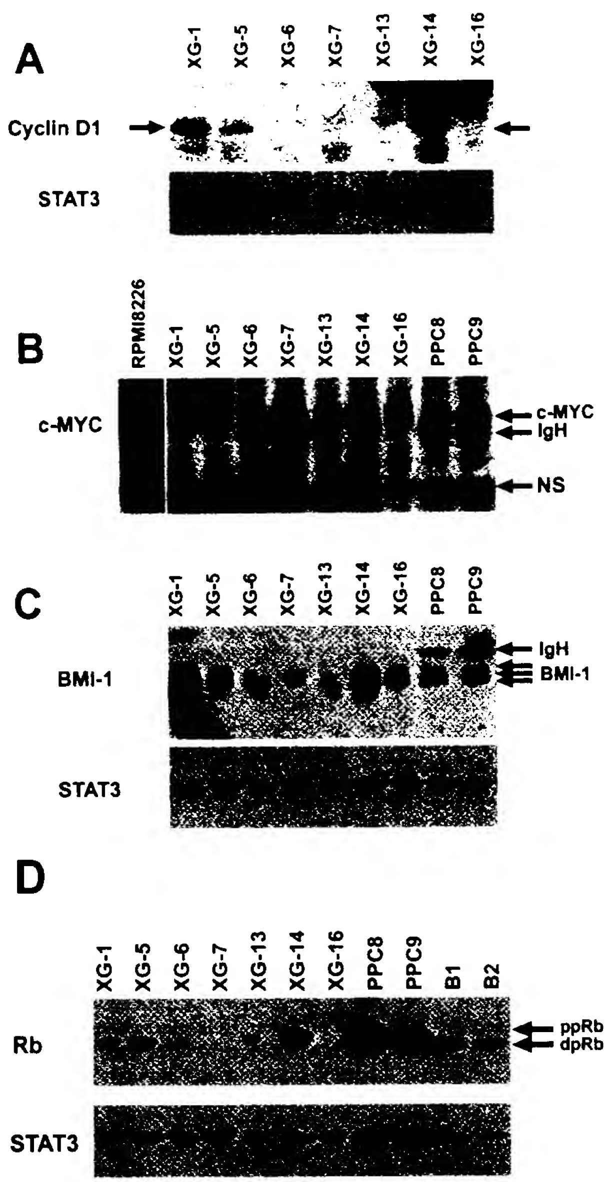

Figure 3 Protein expression analysis of cyclin D1, c-*myc*, BMI-1 and Rb in plasma cells. Protein expression analysis of cyclin D1 (**a**), c-*myc* (**b**), BMI-1 (**c**) and Rb (**d**) in HMCLs, two PPC samples (**b–d**) and two purified B lymphocyte samples (**d**) by WB. STAT3 expression (**a**, **c–d**) or a nonspecific band (**b**) is showed as a loading control. Results of one experiment out of three are shown. NS, nonspecific; pprb, phosphorylated Rb; dprb, dephosphorylated Rb. In PPC, a 55 Kd band was usually detected due to the crossreaction of the polyclonal goat anti-mouse IgH with human IgH. This band is not detected in MM cells that produce a monoclonal Ig at a low level compared to normal plasma cells (Klein *et al.*, 1995)

in MM is a well known feature of malignant plasma cells (Pettersson *et al.*, 1992). Since the balance between pro- and anti-apoptotic members of the Bcl-2 family participates in the cell fate decision between survival and apoptosis, modifications of Bcl-2 and BIK expression are likely linked to an enhanced survival of malignant plasma cells. In addition, several genes whose products have been described as conferring resistance to apoptosis are also up-regulated in MM samples: IER3, heat shock protein 27 (HSP27), and c-IAP2. IER3 is a NF-κB-induced protein protecting from TNF-α- or FAS-induced apoptosis (Wu *et al.*, 1998). HSP27 is a heat shock protein whose over-expression protects against various apoptotic stimuli, including growth factor removal, at least partly by interacting with cytochrome *c* and preventing cytochrome-*c*-mediated activation of procaspase-9 (Bruey *et al.*, 2000). An up-regulation of HSP27 has also been observed in prostatic carcinoma and correlates with a poor clinical prognosis (Cornford *et al.*, 2000). c-IAP2 is a member of the IAP family, which has anti-apoptotic properties by inhibition of caspase. Collectively, we found an up-regulation of genes in myeloma samples whose protein products should cooperate to increase myeloma cell survival and drug resistance.

Altered expression of transduction genes in myeloma samples

ABL tyrosine kinase The expression of the tyrosine kinase ABL, involved in the Philadelphia chromosome in chronic myeloid leukemia, is up-regulated in myeloma samples. This is of particular interest since a constitutive ABL and *myc* activation can induce plasmacytomas in BALB/c mice (Largaespada *et al.*, 1992). We took advantage of the recently developed ABL kinase inhibitor STI 571 to test whether ABL kinase activity is important in MM cell survival or proliferation. We found that the STI 571 inhibitory concentration necessary to obtain a 50% inhibition in MM cell proliferation (IC50) is rather high (5 μM in XG13 to 20 μM in XG1) compared to the BCR-ABL expressing cell line K562 (0.5 μM) (data not shown). As a potential link between ABL and gp130 IL-6 transducer pathways through STAT3 phosphorylation was suggested in murine ABL/myc plasma cell tumors (Hilbert *et al.*, 1996), we evaluated the combined effect of STI 571 and anti-IL-6 antibodies on myeloma cell proliferation. When used alone, an intermediate concentration of STI 571 (3 μM) or of the B-E8 anti-IL-6 antibody (0.03 μg/ml) resulted in a minor decrease in myeloma cell proliferation (36% and 26%, respectively), whereas the combination of STI 571 (3 μM) and B-E8 antibody (0.03 μg/ml) induced a marked (59%) and significant ($P=0.0004$) inhibition (Figure 4a). Thus, when the available IL-6 signal becomes limiting, the kinase activity of ABL turns out to be essential. As a first clue for the molecular explanation of this cooperation between these two signaling pathways, we observed that IL-6 stimulation induced ABL phosphorylation in myeloma cells (Figure 4b). This is the first model in which IL-6 was shown to induce ABL phosphorylation. Further studies are necessary to understand the role of ABL in the myeloma cell survival mediated by gp130 IL-6 transducer activation. We have shown that anti-IL-6 mAb can block myeloma cell proliferation in patients with terminal disease (Klein *et al.*, 1991). More recently, we have shown that these anti-IL-6 antibodies might improve

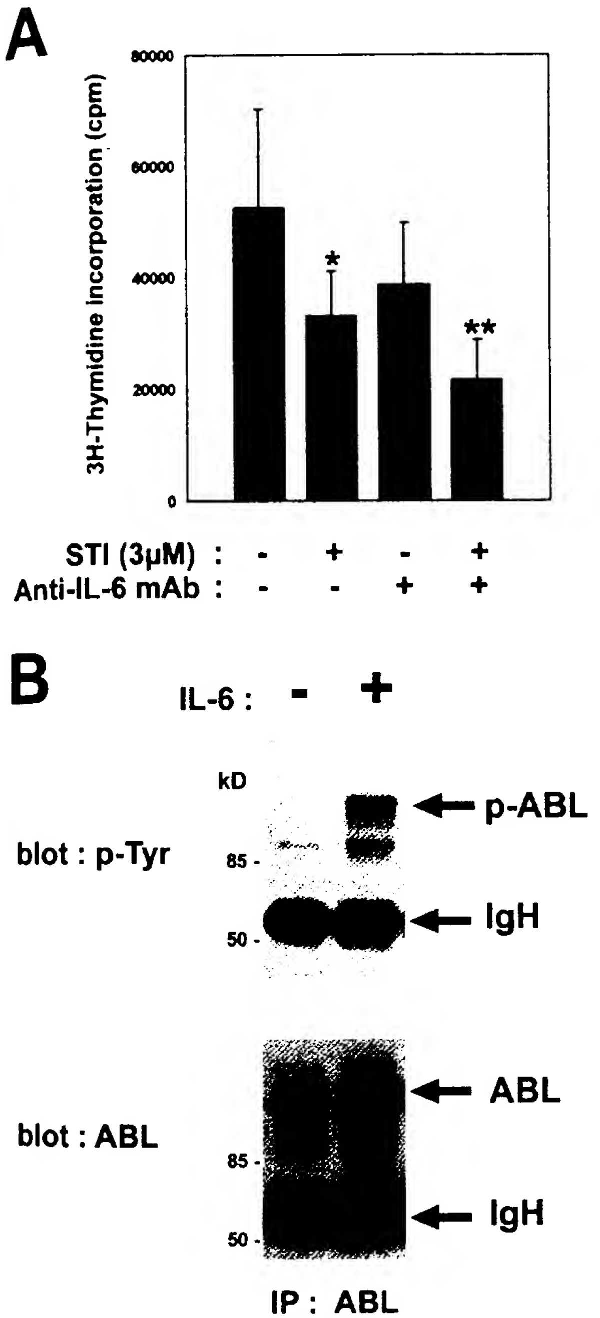

Figure 4 Myeloma cell growth inhibition by STI571 and ABL phosphorylation by IL-6. (**a**) The IL-6-dependent XG-6 HMCL was cultured in medium containing IL-6 for 4 days and in the presence of 3 μM of the ABL kinase inhibitor STI571 or 0.03 μg ml of the B-E8 anti-IL-6 antibody as indicated. Results are tritiated thymidine incorporation mean values $\pm$ s.d. of six wells and are representative of three independent experiments. *Significantly lower values than control ($P = 0.01$); ** Significantly lower values than control ($P = 0.0004$), than STI 3 μM alone ($P = 0.01$), and than B-E8 0.03 μg ml alone ($P = 0.009$). Statistical analyses were carried out using a Mann–Whitney test. (**b**) The IL-6-dependent XG-14 HMCL was cultured without IL-6 for 24 h and restimulated with IL-6 (20 ng ml) for 10 min. Anti-ABL immunoprecipitates were resolved by 7.5% SDS PAGE, and phosphorylation of ABL was detected by immunoblotting with an anti-phosphotyrosine antibody. Blots were stripped and reprobed with an anti-ABL antibody

the overall survival of patients treated with high-dose chemotherapy (unpublished results). The present data suggest that the ABL kinase inhibitor STI 571 could have clinical benefit in combination with anti-IL-6 mAb, at least in the patients with a demonstrated overexpression of the ABL gene in myeloma cells.

Tyro3 receptor tyrosine kinase (RTK) family In a previous study with ATLAS macroarrays, RT–PCR, and WB, we found an overexpression in myeloma cells of Tyro3, an oncogenic receptor tyrosine kinase (RTK) (De Vos *et al.*, 2001). Tyro3 belongs to a family of three RTK members, Tyro3 (also termed Rse, Sky, Tif, or Dtk), MER (also termed Eyk) and Axl (also termed Ufo or Ark), which share the same ligand, GAS6. Likely due to a lack of sensitivity of the Tyro3 probes in the HuGeneFL chip, we only observed a significant expression of Tyro3 by Affymetrix microarray in the XG-6, -7, and -14 HMCLs (Figure 2), which are the samples expected to have the highest Tyro3 expression according to our previous immunoblot data (De Vos *et al.*, 2001). However, we observed that a second member of the tyro3 RTK family, MER, was up-regulated 5.1-fold in MM samples compared to PPC. The third tyro 3 RTK family member, Axl, was not included in our 3874-probe set list. Thus, using various techniques, we found that two members of the tyro 3 TRK family were overexpressed in myeloma cells compared to normal plasma cells. These findings may be of importance because tyro3 is an oncogene in murine fibroblasts and we recently found that both normal and tumor plasma cells highly express GAS6, the common ligand of the Tyro3 RTK family, as compared to B cells (Tarte *et al.*, 2002). Studying the role of an autocrine GAS6 Tyro3 receptor family loop in the function of myeloma cells is problematic due to the lack of commercially available reagents.

Other transducing elements RhoC is a small GTPase that is a transforming oncogene for human mammary epithelial cells. Its overexpression is associated with inflammatory breast cancer and with metastasis formation in melanoma (Clark *et al.*, 2000; van Golen *et al.*, 2000). Our data point to a 8.5-fold increase in RNA expression in malignant plasma cells as compared to their nonmalignant counterpart. To our knowledge, RhoC has never been related to myeloma biology. Its high expression could contribute to inflammatory bone marrow and the high level of tumor dissemination in a majority of MM cases at diagnosis. We also note a higher expression of the NFKB2 gene in myeloma samples. NFKB2 is a member of the REL/NFKB family of transcription factors that was first cloned as an oncogene involved in a translocation with the IgH locus in lymphoid malignancies, including myelomas (Neri *et al.*, 1991). Two genes coding for pre-B cell proliferation enhancers were found up-regulated in myeloma samples: stromal interaction molecule 1 (STIM1) and pre-B-cell colony-enhancing factor (PBEF). These factors increase the IL-7-dependent proliferation of pre-B cells and could therefore play a role in B-

lymphoid malignancies, including MM (Oritani and Kincade, 1996; Samal *et al.*, 1994). The most over-expressed myeloma gene (63 times higher in MM samples than in PPC) is frizzled related B gene (FRZB), an inhibitor of the Wnt pathway. This finding confirms our previous study with ATLAS macroarrays and RT PCR (De Vos *et al.*, 2001).

Altered expression of metabolism genes in myeloma samples

Cystathionine beta synthase (CBS) The oligonucleotide microarray analysis shows that the CBS gene, coding for an enzyme involved in methionine metabolism, is increased 16-fold in myeloma samples. The Affymetrix data were confirmed by RT–PCR (Figure 5a). Due to its localization on chromosome 21, CBS expression is increased in Down syndrome (DS) associated with trisomy 21, and this CBS overexpression could account for the higher sensitivity of DS acute myeloid leukemia blasts to ara-C (Taub *et al.*, 2000). Interestingly, we found a significant correlation ($P=0.04$) between CBS overexpression and ara-C sensitivity in our six IL-6-dependent myeloma cell lines (Figure 5b–d). XG-1 cells were especially highly sensitive to ara-C with an IC50 around 0.6 nM, 10 000-fold lower than the plasma ara-C concentration achievable *in vivo* with clinical protocols (Taub *et al.*, 1996). This observation encourages reconsideration of the use of ara-c in the treatment of patients with MM. This drug did not show any clinical benefit in this disease while its toxicity was high (Kantarjian *et al.*, 1984). However, as the fraction of proliferating myeloma cells is low in patients with MM, a continuous administration mode could be interesting to induce an antitumor effect, at least in the subset of MM patients with CBS gene overexpression.

Phosphofructokinase isoform (PFKM) The PFKM gene is overexpressed 10 times in MM samples. PFKM is a rate-limiting enzyme in glycolysis and should contribute to myeloma cell oncogenesis since cancer cells rely on glycolysis for energy, even in the presence of oxygen, a phenomenon called the Warburg effect. This increased PKFM gene expression and subsequent altered glycolysis in myeloma cells is likely related to the *myc* pathway deregulation discussed above. Indeed, PKFM gene expression can be induced by c-*myc* in fibroblasts (Osthus *et al.*, 2000). The possibility that the PKFM gene is a target of the deregulated c-*myc* pathway in myeloma cells is emphasized by the very tight correlation (Pearson's correlation coefficient $=0.847$, $P=4.10^{-8}$) between c-*myc* and *PFKM* gene expression among the 25 samples.

Other metabolism genes Two genes with increased expression in myeloma samples encode enzymes involved in nucleoside metabolism and their over-expression has been linked to malignancy the nucleoside triphosphate synthesis gene nonmetastatic cells 4 (NME4), which is detected in diffuse astrocytomas but not in normal brain tissue (Huang *et al.*, 2000), and the purine metabolism gene guanine-monophosphate synthetase (GMPS), whose mRNA and protein expression are higher in leukemia cell lines and lymphoblastoid lines than in nontransformed, nonproliferating cells (Hirst *et al.*, 1994).

Genes involved in metastasis or bone remodeling

Secreted protein acidic and rich cystein (SPARC) SPARC is a secreted, multifunctional, extracellular matrix glycoprotein which inhibits cell adhesion and activates MMP2. SPARC expression is linked to an invasive phenotype in glioblastomas and was shown to be necessary for metastasis of melanoma cells in vitro and *in vivo* (Ledda *et al.*, 1997). Thus SPARC could be related to the spreading of MM cells *in vivo*; the observation that this gene is more expressed in our cell lines than in primary myeloma samples is in agreement with this hypothesis (Figure 2).

Legumain and cystatin C Unexpectedly, the gene coding for the cystein protease legumain PRSC1 was increased 12-fold in MM samples as compared to PPC. This gene was recently cloned as an inhibitor of osteoclast activity and bone resorption (Choi *et al.*, 1999), which seems counterintuitive in a bone destructive disease. However, cystatin C, the most potent inhibitor of cystein proteases including legumain, is one of the most highly up-regulated genes in MM samples (50-fold). We confirmed the microarray data by WB and immunohistochemistry for cystatin C and by RT–PCR for legumain (data not shown). One can speculate that legumain expression is a negative feedback loop arising in myeloma bone marrow where excessive osteoclast activity is occurring, but that myeloma cells disrupt this regulatory circuit by expressing cystatin C at a high level.

Cancer/testis tumor antigen genes

The expressions of MAGE A1, A2, and A3 as well as NY-ESO-1 and SSX2 genes were increased in MM samples. These genes have a normal expression restricted to testis but display an aberrant expression in numerous tumor types, including myeloma (Pellat-Deceunynck *et al.*, 2000; Tarte *et al.*, 2002).

Conclusion

Despite recent molecular advances in the knowledge of MM, we are still far from a complete description of the biology of this cancer. This study demonstrates the power of the microarray technology to identify genes deregulated in malignant plasma cells provided that normal plasma cells can be obtained. We used *in vitro* generated plasmablastic cells, whereas in a recently published work, Zhan *et al.* (2002) used bone marrow plasma cells from healthy individuals. A quarter of the genes overexpressed in myeloma cells found in the

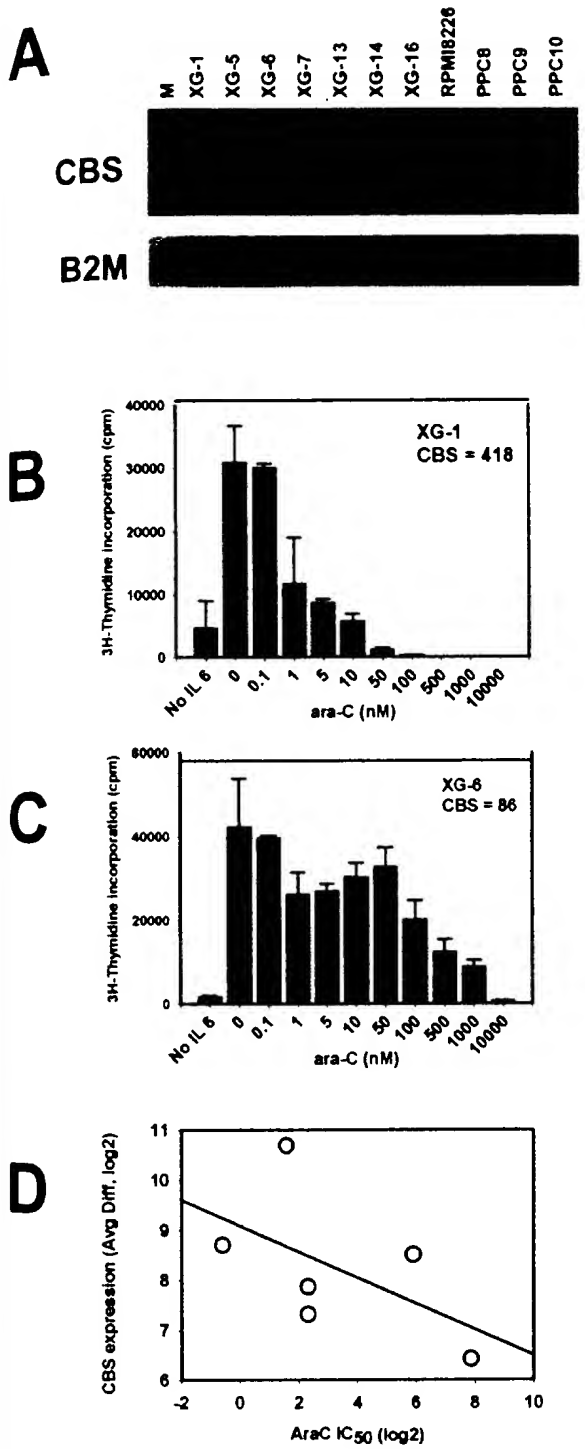

Figure 5 CBS expression and *in vitro* sensibility to ara-C in HMCLs. (**a**) RT–PCR analysis of CBS was performed on cDNA from HMCLs and purified PPC samples. Amplification of β2-M shows the equivalence of the cDNA loading and amplification. Data are representative of two independent RT–PCR assays. (**b** and **c**) *In vitro* sensibility to ara-C in the XG-1 and XG-6 HMCLs was measured by incubating cells with IL-6 and increasing concentration of ara-C for 4 days, before measurement of tritiated thymidine incorporation. One point without IL-6 and without ara-C was included. One experiment representative of three is detailed. Results are mean values ± s.d. of triplicate wells and are representative of three independent experiments. CBS expression (average difference) is detailed for each cell line. (**d**) Relationship between CBS expression levels as measured by oligonucleotide array (average difference) and chemosensitivity to ara-C (IC_{50}) of the IL-6-dependent HMCLs XG-1, 6, 13, 14, 16, and 19 is represented by a dot plot. XG-19 is an HMCL derived from sample PCL1. Values have been transformed into log base 2. The line corresponds to the linear regression (Pearson unilateral correlation coefficient −0.754, $P = 0.042$)

present study were also identified in Zhan's study. The fact that the other genes were not retrieved in common may be explained by distinct statistical methods and different sources of normal plasma cells. Zhan *et al.* (2002) used only statistical tests to select genes differentially expressed between malignant and normal plasma cells. In the current study, we used a combination of one statistical test and the ratio of the mean expression in malignant and normal plasma cells. Indeed, a difference in expression for a given gene between malignant and normal plasma cells should be taken with caution if there is a significant *P*-value but only a minor change in the mean ratio. We chose a minimal ratio of 2 which was established as the approximate limit of sensitivity for Affymetrix oligonucleotides microarrays (reviewed by Su *et al.*, 2002). The source of normal plasma cells is also important. For instance, most proliferation and metabolism genes identified as myeloma genes in the study from Zhan *et al.* (2002) were not found in our study because we used proliferating plasmablastic cells as a normal counterpart. On the other hand, we found several genes upregulated in myeloma cells that were genes also upregulated in mature normal bone marrow plasma cells and that are therefore indicators of plasma cell differentiation. The use of these two sources of normal plasma cells are therefore complementary. Overall our microarray data were confirmed by the differential expression of numerous genes according to published data, by western blot and by RT–PCR. Genes such as CBS and ABL code for enzymes that could be potential therapeutic targets with ara-c or STI571.

Materials and methods

Cell lines

XG-1, XG-5, XG-6, XG-7, XG-13, XG-14, and XG-16 have been obtained in our laboratory and RPMI 8226 from the American Tissue Culture Collection. These HMCLs express cytoplasmic Ig and plasma-cell antigens (Ag) and lack the usual B-cell Ag (De Vos *et al.*, 2001). The HMCLs were grown in RPMI 1640 culture medium supplemented with 10% fetal calf serum (FCS) or X-VIVO 20 serum-free medium (Bio Whittaker, Walkersville, MD, USA) with or without 2 ng/ml of IL-6 (R&D Systems, Minneapolis, MN, USA). All cell lines were free of mycoplasma, as assayed by an ELISA kit (Roche, Meylan, France).

Primary cells

Malignant plasma cells were purified from six patients with MM and three patients with plasma cell leukemia (PCL) after

informed consent. Patient characteristics are summarized in Table 1. One sample of polyclonal plasma cells was obtained from a patient with transient reactive plasmacytosis (RP), as defined by Jego *et al.* (1999). Purification of plasma cells (>95% plasma cells) was performed with the M115 anti-CD138 mAb and anti-mouse IgG1 MACS Microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) as described (De Vos *et al.*, 2001). Seven samples of polyclonal plasmablastic cells were obtained by *in vitro* differentiation of peripheral blood B cells (PPC) from either healthy volunteers or from MM patients using a methodology we recently developed (Tarte *et al.*, 2002). The plasmablastic cells ($CD38^{++}$, $CD20^{-}$) were purified (>99% purity) by high-speed cell sorting using a FACS Vantage Cell Sorter (Becton Dickinson, San Jose, CA, USA). These cells displayed a typical plasma cell morphology on cytological examination, secreted high levels of polyclonal Ig, and lacked the B cell markers CD20, CD21, CD22 and CD23.

cRNA preparation and GeneChip hybridization

RNA was extracted using the RNeasy Kit (Quiagen, Valencia, CA, USA). cRNA was prepared as described previously (Thykjaer *et al.*, 2001), except that total RNA was used as starting material and hybridized to HuGeneFL GeneChip oligonucleotide arrays (Affymetrix, Santa Clara, CA, USA). These arrays contain 7070 sets of oligonucleotide probes representing 6800 full-length human sequences.

Data collection

The probe arrays were scanned at 560 nm using a confocal laser-scanning microscope with an argon ion laser as the excitation source (Hewlett-Packard GeneArray Scanner G2500A). We captured primary data using MAS 4.0 software, resulting in a single raw value for each probe set (average difference) based on the mean of the differences between the hybridization intensity for the perfect match features and the mismatch features for a particular gene. Data from each different array experiment were scaled to 1500 by the Affymetrix GeneChip software in order to account for differences in global chip intensity. Affymetrix files are available as supplementary data (http://www.u475.montp.inserm.fr/BK/SupplementalData.htm). This dataset was floored to 200, since discrimination of expression changes in this low range can not be performed with high confidence.

Statistical analysis

We analysed 3874 genes among the 25 samples, with at least three absolute calls defined as 'present' by the GeneChip software. In order to evaluate how the 25 samples were grouped together according to the similarity of their gene expression profiles, we used two separate unsupervised clustering algorithms: a hierarchical clustering with the average linkage method and an uncentered correlation, and principal component analysis (PCA). For hierarchical clustering, data were log transformed, median centered, and processed with the CLUSTER and TREEVIEW software packages (Eisen *et al.*, 1998). For PCA, data were log transformed, median centered, and processed with the J-Express 1.1 program (Dysvik and Jonassen, 2001). Given the high computational task required for this analysis, we reduced the gene number by selecting the 1500 genes with the highest variation coefficient among the 25 samples. The first three principal components retained 46.6% of the total variance of this dataset. We then determined the genes whose expression significantly ($P \leq 0.05$) varied between normal and malignant plasma cells using the nonparametric Mann–Whitney test. We retained only genes that had a ratio of mean values between these two categories ≥ 2 or ≤ 0.5. The Mann–Whitney test, as well as the Pearson and Spearman correlation calculations, were performed with the SPSS software (SPSS Inc., Chicago, IL, USA).

Western Blot

Western blots (WB) were done as previously described (de Vos *et al.*, 2001). Anti-cyclin D1 monoclonal antibody was purchased from Upstate Biotechnology (Lake Placid, NY, USA), anti-Rb from Pharmingen (San Diego, CA, USA), anti-STAT3 from Transduction Laboratories (Lexington, KY, USA), anti-Mer goat polyclonal antibody from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-BMI-1 F6 monoclonal antibody was a kind gift from Maarten van Lohuizen and anti-c-myc 9E10 monoclonal antibody from Jean-Marie Blanchard.

Reverse transcriptase-polymerase chain reaction

Reverse transcription and PCR were done as previously described (De Vos *et al.*, 2001). The following primers were used for CBS: 5'- AGG ACG GTG GTG GAC AAG TGG TTC AAG AGC AA (sense) and 5'- TGA CCA CCC CGA ACA CCA TCT GCC GCT GAC TG (antisense) (Taub *et al.*, 1999). The size of the CBS PCR product was 656 bp. Annealing temperature was 60°C and the number of cycle 35.

Proliferation assay for myeloma cell lines

Cells were washed twice with RPMI 1640 culture medium, incubated for 3 h at 37°C in RPMI 1640 culture medium and 10% FCS, and washed again. They were then cultured at 10^4 cells per well in RPMI 1640 culture medium and 10% FCS with or without IL-6, and with various concentrations of STI571, provided by Novartis Pharma (Basel, Switzerland), B-E8 monoclonal anti-IL-6 antibody (mAb), provided by John Wijdenes (Diaclone, Besançon, France), or 1-β-D-arabinofuranosylcytosine (ara-C), in 96-well flat-bottomed microplates for 4 days. Tritiated thymidine (0.5 μCi, 25 Ci/mM, NEN, Paris, France) was added for the last 12 h of culture and tritiated thymidine incorporation was determined as reported previously (De Vos *et al.*, 2000). The ara-C concentration that inhibited cell growth by 50% (IC_{50}) is the mean of three independent experiments.

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Original article

SARG: A new human osteosarcoma cell line. Expression of bone markers and of major histocompatibility antigens

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Summary. A new cell line (SARG) was established from a human radiation-induced osteosarcoma (OSA). It showed an epithelial-like morphology with polimorphous and sometimes bizarre nuclei. SARG had an osteoblastic differentiation pattern: almost 100% of the cells were positive for alkaline phosphatase, type I and III collagens and osteonectin. The expression of class I HLA antigens was detectable even after 40 in vitro passages. The expression of MHC antigens was greatly increased after in vitro treatment with interferon gamma (IFN-γ), whereas interferon alpha (IFN-α) and tumor necrosis factor alpha (TNF-α) increased the expression of class I antigens, but not of class II antigens. SARG was tumorigenic after subcutaneos injection in nude mice. Experimental metastases were never detected.

Key words: osteosarcoma, MHC antigens, interferons, tumor necrosis factor-alpha

Introduction

Several cell lines have been established from human OSA [1–7], but the phenotypic pattern and the ability to differentiate along the osteoblastic pathway of many OSA cell lines have not been sufficiently characterized [1, 3–5]. Furthermore, most of them have been maintained in vitro for many years; progressive changes have likely occurred during long-term maintenance in vitro, therefore some of human OSA cell lines may have lost the original features.

We report a new cell line (SARG) derived from a human radiation-induced OSA. Its recent origin makes it a good model for the study of the effects of cytokines with potential therapeutic use on OSA cells. As IFNs have been suggested to influence OSA differentiation [8], we have investigated the ability of IFNs and TNF-α to modulate the osteoblastic differentiation in vitro and the HLA expression of SARG cells.

Materials and methods

Establishment of the SARG cell line

This cell line was established from a human radiation-induced osteosarcoma in a 24-year-old male with histiocytosis X. The tumour was metastatic at the clinical onset and the patient had no received chemotherapy.

In vitro procedures

Cells were routinely cultured in Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin (GIBCO, Paisley, Scotland) and 10% heat-inactivated Fetal Calf Serum (Biological Industries, Kibbutz Beth Haemek, Israel). Cells were maintained at 37 °C in a humidified 5% CO_2 atmosphere. Doubling time and saturation density were determined by daily harvesting of cells after seeding of 20,000 cells/cm^2. Cloning efficiency was evaluated 7 days after seeding of 8–512 cells/cm^2. Cloning efficiency in semi-solid medium was determined 14 days after seeding 400–4,000 cells/cm^2 in 60 mm dishes. Cells were found to be mycoplasma-free by fluorescence staining with Hoechst 33258 [9]. The analysis of DNA content was performed with a static cytofluorometer (Nikon) after staining with DAPI.

Cytokine treatment

Cells were treated in vitro with IFN-α (10–10,000 U/ml), IFN-γ (5–1000 U/ml), TNF-α (100–10,000 U/ml). Recombinant HuIFN-γ and recombinant HuTNF-α were purchased from Boehringer Mannheim Italia SPA, Milan, Italy. Recombinant HuIFN-α was purchased from Hoffman-La Roche, Basel, Switzerland. Cytokines were added to cultures 24 h after seeding; cells were harvested 144 h later.

Differentiation markers and HLA expression

Osteoblastic differentiation markers were analyzed on cytospins fixed with a methanol-aceton solution (3:7) at −20 °C for 10 min. The alkaline phosphatase intracellular content was determined by a cytochemical method (Sigma Diagnostic, St. Louis, MO, USA). The expression of other markers was evaluated by indirect immunofluorescence using polyclonal antibodies respectively specific for type I and III collagen (Chemicon International, Inc. Temecula, CA, USA) and osteonectin (bON-II, kindly supplied by L. W. Fisher, N.I.H., Bethesda, MD, USA). The percentage of positive cells was calculated out of at least 300 cells.

HLA expression was determined by flow cytometry (FACS SCAN IV, Becton Dickinson, San Jose, CA, USA) after indirect immunofluorescence with the following monoclonal antibodies: W6/32 (anti-HLA A,B,C Sera Lab, Crawley Down, UK); OKDR (anti-HLA-DR, Ortho Diagnostic Systems, Milan, Italy); TEC-antiDC-1 (anti-HLA-DQ, [illegible] Genetics, Milan, Italy); B7/21 (anti-HLA-DP, Becton Dickinson, San Jose, CA, USA).

In vivo studies

Male athimic 4–5 weeks old Cr: nu nu(CD-1)BR mice (Charles River Italia, Como, Italy) were used. Tumorigenicity was determined after intraperitoneal (i.p.) and subcutaneous (s.c.) injection of 27×10^6 cells. Tumor growth was assessed twice weekly. Mice were sacrified 6 months after injection. Experimental metastatic ability was evaluated after injection of 0.5×10^6 cells in a lateral tail vein (i.v.) of untreated mice and of mice treated with a single intraperitoneal injection of cyclophosphamide (CY) (200 mg/Kg, Endoxan Asta, FRG) 4 days before cell injection in order to decrease NK activity [10] and enhance experimental metastasis [11]. Mice were sacrified 2 months after injection. For the assessment of pulmonary metastases, lungs were stained with black India ink.

Results

In vitro characterization. All the classical parameters of the in vitro growth were studied between the 20th and the 40th culture passage after 1 year continuous in vitro culture (Table 1). Morphologically, SARG showed a predominance of epithelial-like mononuclear cells, in addition to giant cells with multilobated nuclei (Fig. 1). The cellular diameter was 21.9 ± 0.4 μm with a range from 9.24 to 34.65 μm. The analysis of DNA content showed a hyperdiploid (near tetraploid) pattern.

Differentiation pattern. SARG showed the classical markers of the osteoblastic lineage: 100% of the cells were positive for alkaline phosphatase, 75% for type I collagen, 65% for type III collagen and 61% for osteonectin. HLA A,B,C and HLA-DR antigens were detectable respectively in 84% and 68% of cells, whereas no expression of HLA-DP and HLA-DQ antigens was found.

Effect of cytokines. The effects of IFN-α, IFN-γ and TNF-α on cell growth are shown in Fig. 2. IFN-γ induced a greater inhibition of cell growth than IFN-α; TNF-α was unable to inhibit cell proliferation of SARG cells. Fig. 3 shows the effects of the three cytokines on the expression of class I and II HLA antigens. All the cytokines enhanced the expression of class I antigens, but only IFN-γ was able to increase class II antigens (HLA-DR). HLA-DP and HLA-DQ were not induced.

The ability of TNF-α to increase the expression of class I HLA antigens indicates that these cells have receptors for TNF-α. The osteoblastic differentiation markers (alkaline phosphatase, collagens and osteonectin) were not modulated by IFN-γ or TNF-α.

Table 1. In vitro patterns of SARG cell line.

Doubling time (hours)	Saturation density (cells/cm²)	Clonal efficiency	Cloning efficiency in semisolid medium
58 ± 4	2.5×10^5	2.7 ± 0.3%	0.5 ± 0.1%

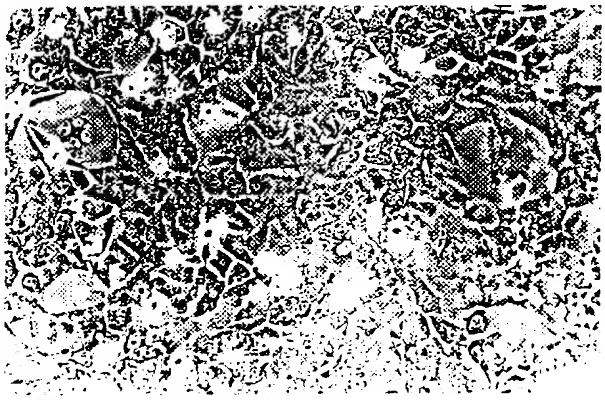

Fig. 1. In vitro morphology of SARG cell line.

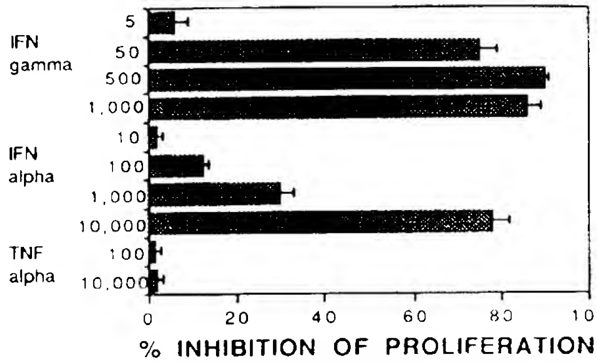

Fig. 2. Inibition of SARG cell growth by IFN γ, IFN α and TNF-α after a 144 h in vitro treatment.

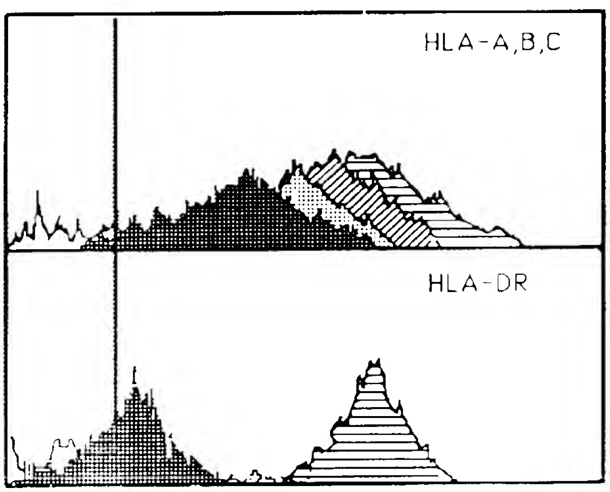

Fig. 3. M[illegible] [illegible] of A.B.C HLA and HLA DR [illegible] express[illegible] SARG cells after a 144 h in vitro treatment [illegible] IFN-α, IFN [illegible] TNF-α. □ = Negative control; ▩ = SARG cell line; □ = IFN [illegible] U/ml; ▨ = TNF α 10,000 U [illegible] = IFN-γ 500 U

In vivo growth and metastasis. SARG was able to grow after subcutaneous injection (27×10^6 cells/mice) even if with a low tumorigenicity and a long latent period (2–4 months) and did not grow in nude mice after intraperitoneal injection (27×10^6 cells/mice). Macroscopic lung metastasis were never observed in untreated mice, whereas a low experimental metastatic ability were found after CY-pretreatment (Table 2).

Table 2. Tumorigenicity and metastatic ability of SARG cells in nude mice.

Site of injection	Pretreatment of mice	Cell dose	Mice with tumor/mice treated	Incidence of lung metastasis
s.c.	None	27×10^6	2/10	
i.p.	None	27×10^6	0/5	
i.v.	None	0.5×10^6		0/5
i.v.	CY	0.5×10^6		2/5

Discussion

SARG is a recent cell line established from a human radiation-induced OSA. It showed the typical markers of the osteoblastic lineage (alkaline phosphatase activity, osteonectin, type I and III collagen). In addition to a high expression of HLA A,B,C antigens, SARG showed low but reproducible levels of HLA-DR antigens expression. The presence of class II HLA antigens in normal osteoblasts has been recently reported [12].

The in vitro treatment of SARG with IFN-α and IFN-γ induced a marked inhibition of cell growth, whereas TNF-α did not have any effect on cell proliferation. Although differentiation markers (alkaline phosphatase, collagens, and osteonectin) were not modulated by IFN-γ and TNF-α in vitro, all three cytokines modulated the expression of class I HLA antigens. Only IFN-γ was able to increase HLA class II (HLA-DR) expression.

Our data indicate that, in addition to its marked cytostatic effect, IFN-γ is able to enhance HLA class I and HLA II antigens expression of OSA cells in vitro. Therefore, it may be considered as an interesting candidate for differentiation therapy of OSA.

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Differentially expressed genes in C6.9 glioma cells during vitamin D-induced cell death program

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Abstract

C6.9 rat glioma cells undergo a cell death program when exposed to 1,25-dihydroxyvitamin D3 (1,25-D3). As a global analytical approach, we have investigated gene expression in C6.9 engaged in this cell death program using differential screening of a rat brain cDNA library with probes derived from control and 1,25-D3-treated cells. Using this methodology we report the isolation of 61 differentially expressed cDNAs. Forty-seven cDNAs correspond to genes already characterized in rat cells or tissues. Seven cDNAs are homologous to yeast, mouse or human genes and seven are not related to known genes. Some of the characterized genes have been reported to be differentially expressed following induction of programmed cell death. These include PMP22/gas3, MGP and β-tubulin. For the first time, we also show a cell death program induced up-regulation of the *c-myc* associated primary response gene CRP, and of the proteasome RN3 subunit and TCTP/mortalin genes. Another interesting feature of this 1,25-D3 induced-cell death program is the down-regulated expression of transcripts for the microtubule motor dynein heavy chain/MAP 1C and of the calcium-binding S100β protein. Finally 15 upregulated cDNAs encode ribosomal proteins suggesting a possible involvement of the translational apparatus in this cell program. Alternatively, these ribosomal protein genes could be up-regulated in response to altered rates of cellular metabolism, as has been demonstrated for most of the other isolated genes which encode proteins involved in metabolic pathways. Thus, this study presents to our knowledge the first characterization of genes which are differentially expressed during a cell death program induced by 1,25-D3. Therefore, this data provides new information on the fundamental mechanisms which participate in the antineoplastic effects of 1,25-D3 and on the machinery of a cell death program in a glioma cell line.

Keywords: Vitamin D; brain; gene expression; apoptosis; differential screening

Abbreviations: 1,25-D3, 1,25-dihydroxyvitamin D3; PMP22, peripheral myelin protein 22; MGP, matrix gamma carboxyglutamic acid protein; CRP, cysteine-rich protein; TCTP, translationally controlled tumor protein; VDR, vitamin D receptor; ORF, open reading frame; UGG, unknown glioma gene; SPARC, secreted protein acidic and rich in cysteine; ON, osteonectin

Introduction

It is presently established that cell death is most often an active process, triggered by precise signals which induce crucial biochemical changes in target cells, resulting in many instances from changes in the pattern of gene expression. However, the precise molecular mechanisms which promote these cell death programs are poorly understood. They may be expected to vary from one cell type to another, while different stimuli have the potential to induce a cell death program by activating independent or overlapping metabolic pathways in the same cell. Previous studies have demonstrated that some genes play a critical role in the triggering of/or progression to cell death. These include nuclear regulatory factors encoded by the *p53* gene (Lowe *et al*, 1993; Hermeking and Eick 1994) and the proto-oncogene *c-myc* (Evan *et al*, 1992), or genes such as *gadd45*, whose expression is controlled by the *p53* protein (Kastan *et al*, 1992; Carrier *et al*, 1994). However, numerous studies evidenced the role of non-nuclear components, such as members of the *bcl2–bax* gene family (Larsen 1994), proteases belonging to the family of interleukin 1-β converting enzyme (Gargliardini *et al*, 1994), or receptors mediating a death signal, such as the TNFα receptor (Yonehara *et al*, 1989), the low affinity NGF receptor (p75) (Barrett and Bartlett 1994) or the Fas/apo-1 receptor (Trauth *et al*, 1989). Many other gene products were reported to be also involved in this process. These include enzymes or structural proteins participating in second messenger transduction cascades, cell cycle progression, cell shape and housekeeping cytoplasmic mitochondrial functions. It has become clear, therefore, that a cell death program cannot be only described by monitoring the expression of some specific markers, but represent profound changes in cell homeostasis, leading to new metabolic situations that eventually promote cell death.

In order to get an overview of the genetic changes occurring during the accomplishment of a cell death program, we have undertaken an approach based on the identification of genes that are differentially expressed in cells that were either in growth-phase, or committed to die. Such genes are potential candidates that are implicated, directly or not, in the cell death mechanism. This methodology was applied in the case of rat C6 glioma cells, whose death was induced by a 24 h-treatment with

1,25-dihydroxyvitamin D_3 (1,25-D3) (Naveilhan et al 1994; Baudet et al, 1996a,b).

1,25-D3 is the most active metabolite of vitamin D. This hormone exerts genomic effects by interacting with a nuclear receptor, referred to as Vitamin D Receptor (VDR), which is structurally related to the receptors of thyroid hormones and retinoic acids (Haussler 1986). 1,25-D3 exerts a tight control on calcemia and bone metabolism. However, the hormone also acts on the immune system. It controls the production of several lymphokines, inhibits T cell proliferation, promotes macrophage differentiation, and has *in vivo* an overall immunosuppressive effect (for review see Thomasset 1994). A growing body of evidence indicates that 1,25-D3 is also active in the central nervous system (CNS). Binding sites of 1,25-D3 were detected in certain neurons and non-neuronal cells (Stumpf and O'Brien 1987, Stumpf *et al*, 1992; Musiol *et al*, 1992), while the mRNA of its receptor, VDR, was identified in the human hippocampus (Sutherland *et al*, 1992) and *in vitro*, in rat primary astrocytes (Neveu *et al*, 1994a). Astrocytes constitute a target for the hormone. They respond to 1,25-D3 by an enhanced production of neurotrophic factors such as NGF (Neveu *et al*, 1994a) or neurotrophin 3 (Neveu *et al*, 1994b). Furthermore, 1,25-D3 enhances the responsiveness of these cells by increasing the expression of the VDR gene. The hormone also promotes its own catabolism by inducing a gene encoding vitamin D-24-hydroxylase (Naveilhan *et al*, 1993). Astrocytes tolerate high concentrations of 1,25-D3, even when cultured in a serum-free medium, that is in the absence of serum vitamin D-binding protein which should limit the concentration of free hormone. This is not the case of malignant C6 glioma cells. Treatment of these cells during 24 h with 10^{-8}–10^{-7} M, 1,25-D3 results 1 week later in the death of a part of the population, provided that cells are maintained in a chemically defined medium (Naveilhan *et al*, 1994). This cytotoxic effect of 1,25-D3 appeared more or less pronounced, depending on the origin of the C6 cells, a fact which is likely to reflect strain variations, observed from laboratory to laboratory on the basis of other criteria (Röser *et al*, 1991; Gubits *et al*, 1992). Therefore, C6 cells were subcloned and a subclone, referred to as C6.9, was selected on the basis of its high susceptibility to the cytotoxic action of 1,25-D3 (Baudet *et al*, 1996a). In C6.9 cells, 1,25-D3 treatment induces accumulation of mRNAs of c-*myc*, *p53* and *gadd45* genes (Baudet *et al*, 1996b). Furthermore, cells are largely protected against the toxic action of 1,25-D3 if they are treated with cycloheximide (Baudet *et al*, 1996b). These data indicate that 1,25-D3 cytotoxicity corresponds to a cell death program, that requires protein synthesis. The interest of this experimental system is strengthened by the fact that glioma form evolutive tumors which are generally fatal in human (for review see Janus *et al*, 1992). Therefore, C6.9 cells appeared adequate to undertake a characterization of genes that are differentially expressed in treated and untreated populations. For this purpose, we have screened an organized library of rat brain cDNA, with complex probes prepared from mRNAs extracted from growing C6.9 cells, and from cells collected 3 days after a 24 h treatment with 1,25-D3. An advantage of organized libraries is that they can be reused, and this permits an overview of a panel of genes whose expression may be compared under different experimental conditions. The present work describes data obtained following the screening of 7680 clones. About 90 of these were found to correspond to mRNAs whose levels do either increase, or decrease, following C6.9 cell treatment with 1,25-D3.

Results and Discussion

Most of the substractive hybridizations or differential screenings performed to characterize genes involved in cell death have been designed to isolate genes specifically expressed during this process. The reasonable assumption that a cell death program is directly controlled by the induction of several genes which are not expressed or expressed at very low levels in exponentially growing cells should not, however, exclude the possibility that cell death might ultimately result from a dysregulation in the expression of some housekeeping genes. The possibility that subtle variations in the levels of expression of this kind of genes could be an important aspect of a cell death program has retained until now little attention. As a first attempt to characterize genes expressed in normal non-apoptotic tissues whose expression is altered during the course of a cell death program, we used a cDNA library derived from Poly(A)$^+$ RNA extracted from adult rat brain. The screening of this library was performed with two complex ^{32}P-labeled cDNAs probes prepared from Poly(A)$^+$ RNA isolated from C6.9 cells 3 days after a treatment of 24 h with either 1,25-D3 or its vehicle. This time point was chosen to allow the detection of changes in RNA expression occurring well before the death of cells, which takes place around day 6 (Baudet *et al*, 1996a), and because a peak in the expression of c-*myc*, *p53*, *gadd45*, IL-6 and VEGF in 1,25-D3-treated C6.9 cells has been recently reported to occur at day 3 (Baudet *et al*, 1996b).

Characterization of the differentially expressed genes

7680 organized clones were screened. In most instances, spot intensity associated with a same clone remained constant. However, signal intensity turned out to diverge after normalization of the spot intensity, by a factor of at least 1.5-fold in 88 clones. These clones were subjected to a confirmatory step based on a Northern blot analysis using total RNA extracted at day 3 from C6.9 cells treated for 24 h with 1,25-D3 or vehicle alone. Thus, Northern blots have been serially hybridized with the 88 plasmids purified from the selected bacterial clones. An example illustrating these different steps of analysis is presented in Figure 1. Figure 1A focuses on a spot (01.11-06) obtained following the screening of the library with complex probes prepared from control (a) or treated (b) cells. Hybridizations with the cloning vector to control bacteria growth are in (c) and (d). The computer analysis of these autoradiograms is presented in Figure 1B. The underexp[illegible] of the gene corresponding to clone 01.11-06 (Figure 1A) identified according to its DNA sequence as being ost[illegible] was further confirmed by

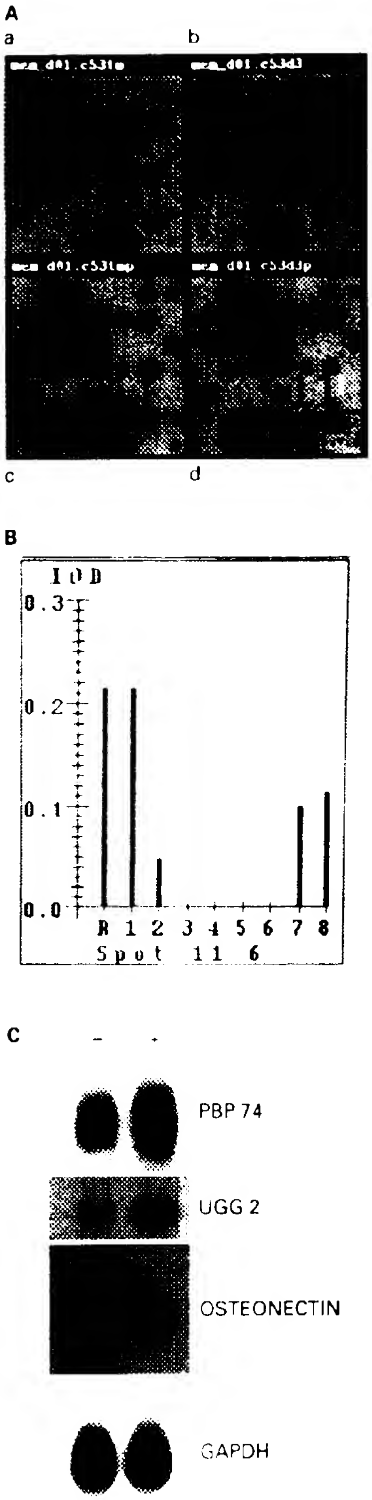

Figure 1 Overview of the different steps of analysis: selection of the spot 01.11-06 which represents the Osteonectin gene. (**A**) Autoradiogram of one of the nitrocellulose membrane containing a part of the cDNA organized library obtained after the labelling with [illegible] probes prepared from control C6.9 cells (a) or 1,25-D3 treated cells (b). Arrowhead indicates the spot 01.11-06 which corresponds to a gene [illegible] expressed. (c) and (d) represent respectively the same membranes labelled with the plasmid alone as control of the bacterial clone growth. (**B**) Computer densitometric analysis of the spot 01.11-06 presented in **A**. R and 1 represent the relative density of the spot 01.11-06 pointed out in **A**(a). 2 represents the relative density of the spot 01.11-06 pointed out in **A**(b). 7 and 8 represent the relative density of the spots pointed out respectively in **A**(c) and **A**(d). (**C**) Northern blot analysis of the [illegible] transcript modulations 3 days after a 24 h treatment with 1,25-D3 (+) or with [illegible] alone (–) and serially labelled with three of the cDNA isolated in [illegible] of this differential screening. These correspond to Osteonectin [illegible] UGG2 (01.15-17), PBP74 (02.48-14) and [illegible] probes

Northern blot analysis (Figure 1C). Northern blot analyses concerning two other genes, which are this time up-regulated in the course of this cell death program, are also presented in Figure 1C. These results were obtained with clones 02.48-18 and 01.15-17, which correspond respectively to a protein involved in cellular senescence called PBP74 or mortalin, and to a novel sequence called UGG2 for Unknown Glioma Gene 2. Using this experimental approach, we were able to select 61 clones encoding sequences corresponding to mRNAs which are effectively regulated during the 1,25-D3-induced cell death program of C6.9 cells. The 17 remaining clones, for which the subsequent Northern blot analyses revealed a different regulation than that expected on the basis of the initial differential screening, were discarded.

Overview of the differentially expressed genes

According to the computer-assisted sequence comparison, these 61 clones can be subdivided into three classes (see also Table 1).

1 Forty-seven clones (approximately 77%) carry inserts with known sequences already detected in rat cells or tissues. It is noteworthy that the length of these inserts is often equal or even longer in 5′ than that already described.
2 Seven clones (approximately 11.5%) carry inserts whose sequences are homologous to mouse, human or yeast genes.
3 Seven clones (approximately 11.5%) carry inserts with novel sequences, including 3 cDNAs containing a potential Open Reading Frame (ORF).

Inserts carried by the 61 clones corresponding to RNA differentially expressed were partially sequenced, and sequences were compared to the GENBANK/EMBL sequence databases. Because of the redundancy of several sequences, the number of different cDNAs isolated after this differential screening was reduced to 45. An overview of these 45 cDNAs is presented in Tables 2 and 3 where they are classified as 'genes induced' (Table 2a), 'genes repressed' (Table 2b), 'homologous sequences induced' (Table 3a), 'homologous sequence repressed' (Table 3b) and 'unknown sequences induced' (Table 4). In addition, data presented in this table give the name of the corresponding genes or proteins, the lengths of each insert (bp), the factor of induction/repression of the corresponding mRNA assessed by Northern blot analysis (d.), and the ACCESS NUMBER in the GENBANK/EMBL sequence or the SWISSPROT/PIR databases.

Table 1 Summary of the results obtained after the differential screening of the adult rat brain cDNA library and the Northern blot analysis

Selected clones after differential screening	88
• Selected clones after Northern Blot analysis	61
• Known sequences	47
• Homologous sequences	7
• Unknown sequences	7
• unknown, with potential ORF	3
• unknown, without ORF	4

Cellular roles of the differentially expressed genes

Another way to present the results of this differential screening is to classify the identified genes with respect to their cellular role. A first attempt of classification is presented in Table 5. In this table genes are grouped into the following six broad categories of biological roles: (1) Cell signalling/Cell communication, (2) Cell structure/Mobility, (3) Cell/Organism defense, (4) Gene/protein expression, (5) Metabolism, and (6) Unclassified. This table does not address to the specific involvements of the different cloned genes in the C6.9 cell death program, but rather gives an overview of the genetic changes, affecting all the general cell functions, which occur in the course of this process. Therefore the discussion has been focused here on the regulation of a dozen of these genes and on the increased expression of ribosomal protein genes.

Osteonectin/SPARC and dynein heavy chain/MAP 1C genes

One of the aims of this work was to try to characterize essential house-keeping gene(s), such as those encoding for vital enzymatic reactions, whose expression could be dramatically decreased in the onset of a cell death program. Among the genes characterized here, three genes are down regulated by more than a factor of five. They encode for Peripheral Myelin Protein (PMP22/gas3) (Spreyer *et al*, 1991), osteonectin/SPARC (Mason *et al*, 1986) and Dynein Heavy Chain/MAP 1C (Mikami *et al*, 1993). The observed down-regulation of osteonectin/SPARC and dynein heavy chain/MAP 1C genes was rather unexpected.

Osteonectin (ON) also known as basement membrane protein (BM40) or Secreted Protein Acidic and Rich in Cysteine (SPARC) displays a high degree of interspecies sequence conservation (Termine *et al*, 1981; Mann *et al*, 1987; Sage *et al*, 1984). It was initially discovered in mineralized tissues (Young *et al*, 1986) and its expression was recently demonstrated in adult rat brain (Mendis *et al*, 1995). The cellular functions of ON/SPARC include effects on matrix deposition and on cell proliferation. An important feature of ON/SPARC is its ability to abrogate the interactions of cells with cytokines such as PDGF or bFGF (reviewed by Lane and Sage 1994). Therefore, the consequence of the down-regulation of ON/SPARC observed here would be to increase cell responsiveness to cytokines. This suggests that the decreased expression of ON/SPARC could be, together with the induction of VEGF and IL-6 expressions (Baudet *et al*, 1996b), a part of the mechanisms observed under stressful conditions to enhance cell viability.

On the other hand, the down-regulation of the Dynein Heavy CHain/MAP 1C (Mikami *et al*, 1993) gene could be directly connected to the mechanisms involved in the course of apoptosis. The Dynein Heavy Chain/MAP 1C encodes the heavy chain of a molecular complex called dynein that has been found to be responsible of intracelular movements associated with microtubules. These include transport of endosomes, lysosomes and the elements of the Golgi apparatus (for review see Vallee 1993; Barton and Goldstein 1996). Its localization to kinetochores is also suggestive of a role in the movement of the chromosomes to the pole. In addition, evidences have been presented demonstrating that dynein plays a role in mitotic spindle formation (Vaisberg *et al*, 1993). Therefore, in view of these functions, this gene fulfills the criteria of a house-keeping gene whose down-regulated expression could induce lethality. This assumption was further reinforced by the fact that loss of function of the dynein light chain has been reported to cause widespread apoptotic cell death in *Drosophila melanogaster* (Dick *et al*, 1996). Experiments with antisense oligonucleotides could help to clarify the effect of a decrease in dynein activity on cell viability. An intriguing point will be to determine whether a reduction of this microtubule motor could be involved in the mechanisms responsible of the margination of clumped nuclear chromatin observed in apoptotic cell.

Matrix Gla protein, β-tubulin, TCP-1-delta, PMP22/gas 3 and PBP74/mortalin genes

Matrix Gla protein (MGP), β-tubulin and PMP22/gas3 correspond to genes already described in other experimental systems to be over-expressed during a cell death program. However, it should be pointed out that PMP22/gas3, which has been associated with the induction of apoptosis in Schwann cells (Fabbretti *et al*, 1995), is down-regulated here. Interestingly, the underexpression of the PMP22/gas3 gene is known to increase the proportion of cells that enter the S+G2/M phases (Zoidl *et al*, 1995), a situation recently found to occur during the course of the cell death program studied here (Baudet *et al*, 1996b).

Matrix Gla (gamma carboxyglutamic acid) Protein (MGP) (Price *et al*, 1987) is a Vitamin K dependent protein and serves as a substrate for the enzyme γ-carboxylase which converts glutamic acid to a γ-carboxyglutamic acid (Price *et al*, 1987). MGP mRNA has been detected in many tissues including brain (Fraser and Price 1988; Hale *et al*, 1988; Price *et al*, 1987; Rice and Price 1994) and the MGP protein is secreted by a large variety of cells in culture (Rannels *et al*, 1993; Fraser and Price 1988; Hale *et al*, 1988). This gene is induced in rat prostate following castration, which causes apoptosis of androgen-dependent prostate cells (Briehl and Miesfeld 1991).

One of the selected cDNA clones encodes the rat β-tubulin (Ginzburg *et al*, 1980). This up-regulation of β-tubulin can be correlated to the enhanced expression of TCP-1-delta (clone 25-13) since this latter protein is a

Tables 2 and 3 Characterization of the analyzed cDNA clones selected after the differential screening of 7680 of the adult rat brain library The data represent the name of the genes induced (2a), repressed (2b), and the homologous sequences induced (3a), repressed (3b) corresponding to the differentially expressed cDNA clones

TABLE 2:
a *Genes induced*

Clone	Frequency	Size of insert (bp)	Putative sequence	d*	Access Number
04.40-05	1	1000	Ribosomal protein S2	+1.5	RO:RRRPS2
03.31-27	1	500	Ribosomal protein S6	+2	RO:RATRPS
02.03-22	2	800	Ribosomal protein S8	+2	RO:RNRPS8
01 34-08	1	700	Ribosomal protein S9	+4.7	RO:RNRPS9
02.10-17	1	600	Ribosomal protein S12	+2.4	RO:RATRPS12
01 40-08	1	600	Ribosomal protein S17	+3.6	RO:RATRPS17
03 35-15	1	600	Ribosomal protein S18	+2.7	RO:RRRPS18A
01 22-21	3	600	Ribosomal protein S25	+2	RO:RRRPS25
03 35-27	4	800	Ribosomal protein L9	+1.9	RO:RRRPL9
03 08-06	1	1000	Ribosomal protein L13a	+1.7	RO:RNRPL13A
01.43-19	1	650	Ribosomal protein L23	+1.5	RO:RRRPL23A
04 36-07	1	600	Ribosomal protein L26	+2.3	RO:RRRPL26
01 05-26	1	560	Ribosomal protein L44	+2	SW:RL44_RAT
03 29-05	1	500	Ribosomal phosphoprotein P1	+1.5	RO:RRRP1
02 06-05	1	500	Ribosomal phosphoprotein P2	+1.6	RO:RRRPP2
02 16-11	1	2500	28S RNA	+2	RNRRNA04
02 29-12	4	1300	Mitochondrial gene 16S RNA	+2.5	MIRNXX
03 37-13	1	850	Proteasome RN3 subunit	+3.3	RO:RATRN3
01 46-28	2	1700	Cysteine-rich protein	+7.1	RO:RNU09567
03.10-29	2	600	Transthyretin	+2.5	RO:RNTTHY
01.22-09	1	1300	Rap 1B	+2.7	RO:RNU07795
03.39-27	2	600	Matrix Gla Protein	+3.8	RO:RATMGP
02.33-10	1	1200	β-Tubulin	+4.3	RO:X03369
02.07-05	1	1100	Phosphoglycerate mutase type B	+2.5	RO:S63233
02.34-24	1	1800	Cytosolic Aspartate Aminotransferase	+2.3	RATCASPAT
02.40-24	1	1100	Cytochrome C Oxydase subunit I	+1.5	RO:S79304
b. *Genes repressed*					
01 10-17	1	1200	Peripheral Myelin Protein 22	−6.8	SW:PM22_RAT
01 11-06	4	1300	Osteonectin	−7.7	SW:SPRC_RAT
01 11-01	1	1400	Dynein Heavy Chain MAP 1C	−5.6	SW:DYHC_RAT
03 30-07	1	1400	Aldolase C	−1.5	SW:ALFC_RAT
02 18-27	2	1800	β subunit s-100 Protein	−3.5	RO:RATs100b

TABLE 3:
a. *Homologous sequences induced*

Clone	Frequency	Size of insert (bp)	Putative sequence	Identity	d	Access Number
03.25-13	1	1300	TCP-1-Delta	100% mouse	+1.6	RO:MUSCHAP
02.42-23	1	1500	KOX30	90% human	+2	PR:HSKOX30
02.48-14	1	1000	PBP74	100% mouse	+2.4	RO:MUSPBP74
02.30-19	1	950	Proteolipid Protein A1	52% yeast	+1.8	SP:PPA1_YEAST
03.22-18	1	900	Translationally Controlled Tumor Protein	100% mouse	+2.3	RO:MML21KD1
b. *Homologous sequences repressed*						
02.13-21	2	1000	Stearoyl-CoA desaturase	90% mouse	−4.4	RO:MUSSCD2
01.26-20	2	2000	Probable E1-E2 ATPase YIL048W	52% yeast	−1.7	SW:YIE8 YEAST

Frequency indicates the number of cDNA clones which encodes the corresponding sequence. d* is the fold of increase (+) or decrease (−) in comparison with the control after densitometric analysis of the Northern blot autoradiogramms. Northern blot analyses have been verified in two different experiments and correlate with the results of the differential screening. ACCESS NUMBER: Number of access in the GENBANK/EMBL sequence or SWISSPROT/PIR databases

member of cytoplasmic chaperonins, which supports the correct folding of β-actin and tubulin (Gao *et al*, 1992; Yaffe *et al*, 1992). It is known that the relative β-tubulin immunofluorescence as measured by FCM in human leukemic T-cell line CCRF-CEM treated by a cytotoxic drug, increased in apoptotic populations compared to untreated cells (Pittman *et al*, 1994). It has also been recently found that in *S. cerevisae*, genetic configurations resulting in an increase in the ratio of β-tubulin to α-tubulin, causes microtubule disassembly and cell death (Archer *et al*, 1995). It remains now to determine whether the increased expression of β-tubulin observed here affects the ratio of the microtubule constituents. Finally the classification of PBP74, also called mortalin, with this set of genes is motivated by its involvement in the determination of cellular senescence (Wadhwa *et al*, 1993).

Table 4 Sequencing analysis and Northern blot analysis of inserts with unknown nucleotide sequences. d' is the fold of increase (+) in comparison with the control after densitometric analysis of the Northern blot autoradiogramms. Northern blot analyses have been verified in two different experiments and correlate with the results of the differential screening

Clone	Particularity	Insert (bp)	d'	mRNA (kb)
Unknown sequences				
UGG1 (01.14-23)	EST	1200	+2.4	3.5
UGG2 (01.15-17)	None	700	+5.5	10
UGG3 (02.03-12)	None	2400	+6.3	1.2
UGG4 (02.22-12)	ORF 52 AA	1000	+2.6	1.2
UGG5 (02.33-08)	ORF 97 AA and signal peptide	2000	+2.2	>10
UGG6 (02.38-23)	ORF 63 AA	1100	+2.1	1.5
UGG7 (03.45-21)	EST	1100	+2.1	1.4/1.8

Table 5 List of the identified genes classified with respect to their cellular role

Genes related to	Identification
Cell signalling/ Cell communication	Transthyretin RAP1B
Cell structure/ Motility	PMP22 β-Tubulin Dynein Heavy Chain MAP1C Matrix Gla Protein Osteonectin Proteolipid protein A1
Cell/Organism defense	PBP74
Gene/Protein expression	Cysteine-rich protein TCP-1-delta Proteasome RN3 subunit Ribosomal proteins (S2, S6, S8, S9, S12, S17, S18, S25, L9, L13a, L23, L26, L44) Ribosomal phosphoproteins (P1, P2) 28S RNA Mitochondrial gene 16S RNA
Metabolism	Cytosolic Aspartate Aminotransferase Cytochrome C Oxydase subunit I Stearoyl CoA Desaturase Aldolase C Phosphoglycerate mutase B Probable E1-E2 ATPase YIL048W
Unclassified	β-subunit S-100 protein TCTP

Cysteine-rich protein and proteasome RN3 subunit genes

These two genes, which have not been associated until now with cell death, are up-regulated by more than a factor of three in C6.9 cells committed to die.

Cysteine-rich protein (CRP) gene encodes a member of a protein superfamily containing a double zinc finger-like motif. The expression of this gene is developmentally regulated in rat brain (McLaughlin *et al*, 1994), but its biological function is not yet understood. In human quiescent fibroblasts, CRP has been shown to be a primary response gene displaying coordinate serum induction with c-*myc* (Wang *et al*, 1992). This finding is interesting in light of recent work showing that c-*myc* gene is overexpressed at day 3 in C6.9 treated with 1.25-D3 (Baudet *et al*, 1996a,b). It is noteworthy that c-*myc* expression may induce apoptosis when combined with a block of cell proliferation (Evan *et al*, 1992). Therefore, data presented here raise the possibility that the coordinated expression of CRP and c-*myc* genes in C6.9 cells cultured in serum-free medium could be directly involved in the cell death program.

cDNA clone 03.37-13 specifies proteasome RN3 subunit (Thomson *et al*, 1993). This protein is one of the numerous components of rat proteasomes, which degrades proteins conjugated to ubiquitine in an ATP-dependent mode (Waxmann *et al*, 1987; Hough *et al*, 1987). This proteasome plays a major role in non lysosomal pathways of protein turnover (Goldberg and Rock 1992). Experimental evidences implicate this proteolytic pathway in the degradation of mitotic cyclins (Deshaies *et al*, 1995), oncoproteins as c-Myc (Ciechanover *et al*, 1991), or the tumor suppressor protein p53 (Scheffner *et al*, 1990). It is noteworthy, in this respect, that an overall increase in the amounts of the 26S proteasome complex takes place during programmed cell death (Dawson *et al*, 1995). This suggests that an increased expression of this gene could be detected in the course of other cell death programs.

Ribosomal protein and translationally controlled tumor protein genes (TCTP)

An important feature of this differential screening is the high number of clones encoding ribosomal proteins, which account for about one third of all selected cDNAs. The various ribosomal protein genes are widely dispersed in the genome. They are transcribed at very similar rates owing to the equivalent strenghts of their promoters (Hariharan *et al*, 1989), and a coordinate regulation, at various levels of gene expression, operates to maintain the proper stoichiometry of the ribosomal components (Mager, 1988). In yeast, most of the ribosomal protein gene promoters contain one or two sites for a global regulator, RAP1p (Klein and Struhl 1994; Kraakman *et al*, 1993), and the transcription of ribosomal proteins is coordinated with rRNA synthesis (Warner, 1989). Conserved motifs where also found in chicken (Maeda *et al*, 1993) or mouse (Genuario *et al*, 1993) ribosomal protein genes, and a coregulation of a set of ribosomal proteins and rRNA was already described in rat liver after a glucocorticoid stimulation (Flusser *et al*, 1989). As we know that these genes are very conserved throughout species, the existence of such a global regulator or common motif of regulation probably exists in the rat genome. The overexpression of several ribosomal protein genes can be compared to the up-regulation of TCTP, another gene isolated in the course of this differential screening, since this gene shares a feature observed on the 5' terminus of the transcripts of some mouse ribosomal protein genes, a run of Ts followed by a GC-rich segment (Chitpatima *et al*, 1988; Wagner and Perry 1985). This finding raises the possibility that this conserved sequence could be a target for a transcriptional factor induced during this cell death program. Electrophoretic mobility shifts assays experiments will help to clarify this

point. Another intriguing feature of TCTP mRNA is that it occurs as untranslated mRNP particles unable to interact with the translation machinery in mouse tumor cell line, in spite of an open reading frame encoding a protein of 172 amino acids (Chitpatima *et al*, 1988).

It is presently not clear whether the differential expression of several ribosomal protein genes plays an active role in this cell death program, or if it only reflects the metabolic disturbance occuring during this cell death process. As outlined up-there, the assembly of functional ribosomes requires precise stochiometries and coordinate expression of more than 80 genes. Therefore, any dysfunction of ribosome assembly could theorectically lead to formation of aberrant and toxic particles. Interestingly, this overexpression of several ribosomal protein genes is not observed in astrocytes treated with 1,25-D3, which have been previously reported to respond to 1,25-D3 by the induction of several genes including VDR, but fail to induce a cell death program (data not shown). The involvement of the translational machinery in cell death has been limited until now to the synthesis of novel proteins at the onset of apoptosis. Nevertheless, our results suggest that a possible link between ribosomes and cell death could exist. Consistent with this hypothesis is the existence of ribonucleoprotein complexes composed of 5S RNA and ribosomal L5, mdm2 and p53 proteins, whose function is however still unknown (Marechal *et al*, 1994).

Unknown sequences (Table 5)

Sequence analysis of seven clones did not reveal known or homologous sequences. They were then called Unknown Glioma Gene (UGG). Three of them present a putative Open Reading Frame (ORF) which could suggest the translation of polypeptides composed of at least 52 amino-acids for the clone UGG4, 63 amino-acids for the clone UGG6, and 97 amino-acids for the clone UGG5. The finding of a putative signal peptide suggests that the potential protein corresponding to the clone UGG5 could be secreted.

Two other unknown sequences have already been indexed in the Gene Databank as human EST after a randomly and automatized sequencing. The clone UGG1 was reported to be a non-codant sequence and the clone UGG7 has not been further analyzed.

The extensive study of these genes will be of great interest to make up the synthetic scheme proposed in Figure 2.

In spite of active research, little is known on the biochemistry and on the genetics which control cell death. In a preliminary approach to solve the puzzle of a cell death program in a rat glioma cell line, we have characterized 61 genes whose expression is differently regulated in the course of this process. In a previous work, we reported that this process was also associated with increased expression of genes such as c-*myc*, *p53*, *gadd45*, VEGF and IL6 (Baudet *et al*, 1996b). A synthetic picture of the possible interaction of these corresponding proteins in the course of the 1,25-D3-induced-C6.9 cell death program is presented in Figure 2. However, it is important to mention that all the data of this differential screening concern mRNA levels and do not account for possible translational or post-translational events. Several differentially expressed genes which have not been extensively discussed here have been reported there. These include Rap1B, which belongs to the Ras family, and S100β and aldolase C genes. An interesting feature concerning these two later genes, which are down-regulated here, is that their corresponding proteins interact together (Zimmer and Van Eldick 1986). In addition, disruption of the microtubular cytoskeleton is known to cause a specific reduction in the level of S100 protein mRNA in C6 cells (Dunn *et al*, 1987). S100 protein is a low molecular weight, Ca^{2+}-binding protein (Pritchard and Marston 1991), then a reduction of S100β mRNA could result in a decreased calcium-buffering potential of the cells, and increased susceptibility to the excessive calcium loading, often reported to occur during programmed cell death. A major task will be now to determine how all these variations in gene expression are integrated in a common cell death mechanism. In this regard, the possibility exists that, in view of the biological importance of cell death, several distinct and independent programs, each of them sufficient to induce cell death, could be active simultaneously in the same cell during a given event of cell death program. This could further complicate our ability to elucidate the individual components of a specific cell death program event.

Materials and Methods

Cell culture

Clone C6.9 used in this study was isolated from the rat glioma C6 cell line (Benda *et al*, 1969; Baudet *et al*, 1996a) by the method of limiting dilution in microtiter plates. The microtiter plates were screened for wells containing single cells 24 h after seeding. Cells were maintained in F12 medium supplemented with 10% fetal calf serum and were used between passages 5–20. For the experiments, cells were rinsed once with PBS and incubated for 24 h in a serum-free medium consisting of F12 medium supplemented with insulin (2.5 μg/ml), transferrin (2.5 μg/ml) and selenium (2.5 ng/ml), penicillin (100 units/ml), streptomycin (100 μg/ml) and amphotericin B (0.25 μg/ml). Then, 1,25-D_3 was added to the cultures at 5×10^{-8} M and left during 24 h. At the end of this incubation period, media were replaced by a serum-free medium devoided of 1,25-D_3. Control cultures were treated with vehicle alone (ethanol).

Preparation of complex cDNA probes for differential screening

Total RNA was extracted following the LiCl/urea method (Auffray and Rougeon 1980) from C6.9 cells harvested 3 days after a 24 h treatment with 5×10^{-8} M of 1,25-D3 or ethanol, and submitted to chromatography on oligo(dT)-cellulose (Aviv and Leder 1972). Two cDNA complex probes were simultaneously prepared from 1 μg of poly(A)-rich mRNAs using oligo(dT)12-Apa1 primer adapter, in presence of [^{32}P]dCTP (with a specific activity of 3000 d.p.m./ng cDNA) and SuperScript II reverse transcriptase (B.R.L., Charbonnière, France). cDNAs obtained were [illegible] quantified and cDNA patterns verified on denaturing gels. Then the two cDNA probes corresponding to C6.9 cells treated or not with 1,25-D3 were labeled with high specific

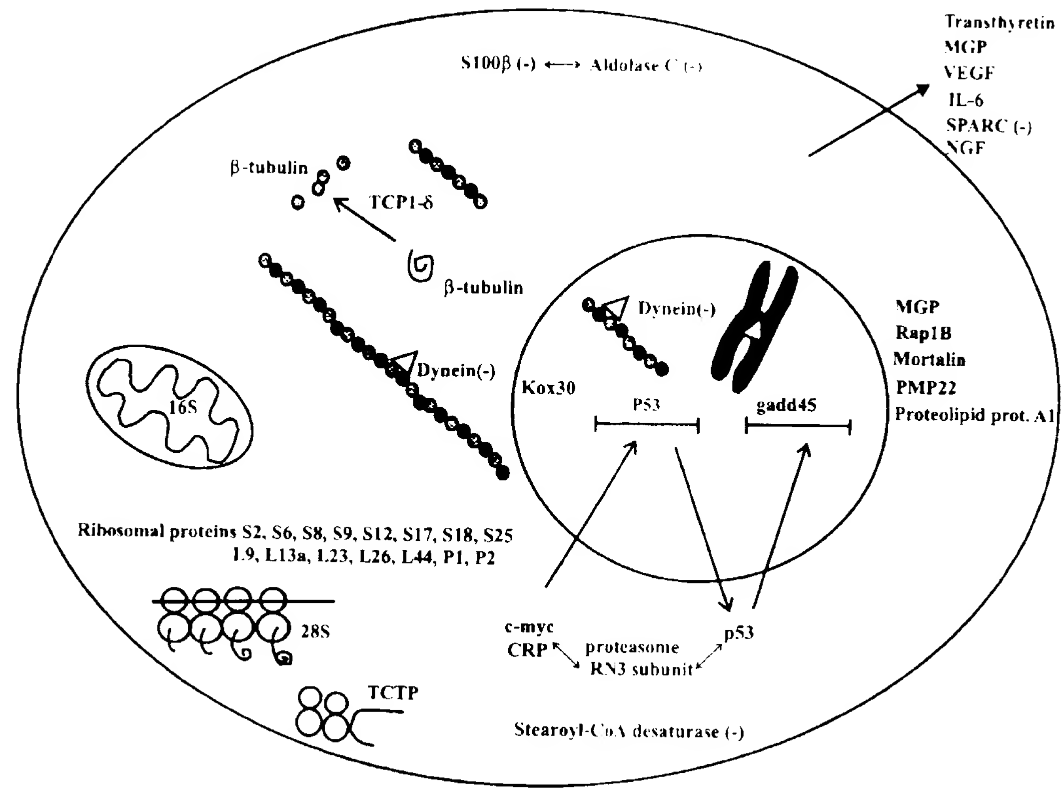

Figure 2 Schematic representation of the localization and of the possible interactions between the proteins corresponding to genes differentially expressed during C6.9 cell death program. ↔ illustrates physical interactions

radioactivity using the random primer DNA Labelling System for BRL (2×10^9 d.p.m./μg). Non-incorporated nucleotides were separated by two successive chromatographic steps on a Biogel P10 (BioRad, Ivry sur Seine, France) column.

cDNA library and high density filters

The precise procedure is described in Perret *et al* (submitted for publication). Briefly, the cDNA library was prepared from rat brain mRNA followig the primer adapter method described by Caput *et al* (1986). The cDNA library was constructed using the cloning vector pT7T3 18 (Pharmacia) and transformed by electroporation in highly competent DH5α bacteria (10^{10} c.f.u./mg DNA). The cDNA library was organised in plates of 1536 well-dishes using a modified fluorescence activated cell sorter (FACS IV Becton Dickinson). After bacterial growth, each plate was replicated onto several identical nylon membranes (Filter Pall) with a Biomek 1000 workstation. After overnight incubation at 37°C, bacteria on membranes were lysed and treated as described by Nizetic *et al* (1991).

Hybridization and differential screening analysis

Two replicas of five 1536 well-dishes were hybridized for 48 h at 42°C with complex probes prepared from C6.9 cells treated or not with 1,25-D3. For each filter 50 ng of the probe labelled as described above was added to 5 ml of hybridization mix (formamide 50%, Denhardts 5×, SDS 0.1%, 6×SSC, DNAss 100 μg/ml). After hybridization, filters were washed once in 2×SCC, 0.1% SDS at room temperature and then successively in 0.1×SSC, 0.1 SDS at 42°C for 30 min and 65°C for 60 min.

The hybridized filters were revealed in a phosphorimager screen system (Molecular dynamics), the files were transferred to SUN Sparc 10 workstation and comparisons were performed using BioImage program (Millipore, USA).

Then, each filter was re-hybridized by a radiolabelled oligonucleotide recognizing pT7T3 18 vector to determine the quantity of plasmid on filters. The normalisation of the spot intensity by assaying plasmid quantity permitted to eliminate artefactual variations.

DNA sequencing and computer analysis

For each of the selected clones, around 300–500 bp were sequenced using a dyed terminator sequencing kit from Applied Biosystem (ref: 402122). The sequences thus obtained, corresponding to the 5' ends of the coding strands of the inserted cDNAs, were compared with the whole set of known nucleic sequences (GENBANK/EMBL sequence databases) in order to ascertain whether they are already known or if they are homologous or related to known sequences.

For each unknown sequence, the presence of an Open Reading Frame (ORF) was investigated and the longest ORF was compared with the whole set of protein sequences stored in SWISSPROT/PIR databases.

RNA isolation for Northern blot analysis and cDNA probe preparation

Total RNA was extracted following the LiCl/Urea method (Auffray and Rougeon 1980) from C6.9 cells treated or not with 1,25-D3, as described above. Identical amounts of glyoxal-treated RNA were then subjected to electrophoresis, transferred to a Hybond N membrane (Amersham, Les Ulis, France) and hybridized as previously described (Wion *et al*, 1991) with cDNA inserts carried by the plasmids of each bacterial clone selected in the library. Plasmids were purified according to the plasmid mini-procedure from Promega (Charbonnière, France), and labelled with [^{32}P]dCTP by random priming as described above. Standardization of RNA loading was routinely controlled by hybridization of the blots with a GAPDH cDNA (Fort *et al*, 1985). Blots were subjected to autoradiography, and autoradiograms were analyzed by densitometric tracing. Probes giving a signal differing by a factor of at least 1.5-fold, when hybridized with RNAs extracted from control or 1,25-D3 treated cells, were selected for further analyses.

Evaluation of the length of inserts

Each selected bacterial clone was cultured overnight. Bacteria were lysed under alkaline conditions, and plasmidic DNA was purified with the Qiagen KIT (COGER, Paris, France). Electrophoresis on a 1% analytical gel permitted the estimation of the size of inserts, after a double digestion with two different restriction enzymes: *Hind*III and *Bam*HI, this in comparison with a lane containing a size ladder made of Marker 2 (λ/*Hind*III, *Eco*RI double digestion) from EUROGENTEC (Angers, France).

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Report

Doxycycline-inducible expression of SPARC/ Osteonectin/ BM40 in MDA-MB-231 human breast cancer cells results in growth inhibition

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Summary

SPARC (secreted protein acidic and rich in cysteine)/BM40/Osteonectin is a matricellular protein with multiple effects on cell behaviour. *In vitro*, its major known functions are anti-adhesive and anti-proliferative, and it is associated with tissue remodelling and cancer *in vivo*. SPARC is overexpressed in many cancers, including breast cancer, and the effects of SPARC seem to be cell type-specific. To study the effects of SPARC on breast cancer, we transfected SPARC into the MDA-MB-231 BAG, human breast cancer cell line using the Tet-On inducible system. By western analysis, we found low background levels in the MDA-MB-231 BAG and clone X parental cells, and prominent induction of SPARC protein expression after doxycycline treatment in SPARC transfected clones X5, X21, X24 and X75. Induction of SPARC expression did not affect cell morphology or adhesiveness to collagens type I and IV, but it slowed the rate of proliferation in adherent cultures. Cell cycle analysis showed that SPARC slowed the progression to S phase. Doxycycline induction of SPARC also slowed the rate of monolayer wound closure in the cultured wound healing assay. Thymidine inhibition of proliferation abrogated this effect, confirming that it was due to anti-proliferation rather than inhibition of migration. Consistent with this, we were unable to detect any differences in migration and Matrigel outgrowth analysis of doxycycline-stimulated cells. We conclude that SPARC is inhibitory to human breast cancer cell proliferation, and does not stimulate migration, in contrast to its stimulatory effects reported for melanoma (proliferation and migration) and glioma (migration) cells. Similar growth repression by SPARC has been reported for ovarian cancer cells, and this may be a common feature among carcinomas.

Abbreviations: DOX: doxycycline; FCS: fetal calf serum; rSPARC: recombinant secreted protein acidic and rich in cysteine; kDa: kilo-Dalton; BAE: Bovine aortic endothelial; ECL: enhanced chemiluminescence; TCA: trichloroacetic acid; SRB: sulforhodamine B; rpm: revolutions per minute; FACS: Fluorescence activated cell sorter; VEGF: vascular endothelial growth factor; RT-PCR: reverse transcription polymerase chain reaction

Introduction

SPARC (secreted protein acidic and rich in cysteine) is a secreted, 32 kDa glycoprotein that is principally produced by cells of mesenchymal origin. It is also known as BM40 [1], since it is a component of basement membrane matrix, and Osteonectin, since it was isolated through its' affinity for bone matrices [2]. SPARC is a matricellular protein; it bridges the gap between cells and the extracellular matrix (ECM) to mediate cell–matrix interaction, but does not serve a primary structural role [3]. SPARC expression in the adult is largely limited to tissues undergoing repair or remodelling such as bone growth and wound healing, and elevated expression of SPARC is found in many pathologies [4].

In vitro, the two major effects of SPARC on mesenchymal cells are anti-adhesive and anti-proliferative. Exogenous SPARC added to cells in culture has been shown to induce a round morphology in confluent

monolayers of bovine smooth muscle cells, fibroblasts and endothelial cells, and to maintain the rounded morphology of newly plated fibroblasts by inhibiting their spreading [5]. Exogenous SPARC inhibited the incorporation of [^{3}H]-thymidine by as much as 90% in synchronized cultures of bovine aortic endothelial cells [6]. However, the effect of SPARC on proliferation is cell type-specific. It does not affect the growth of melanoma [7] or prostate cancer cell lines [8], but reduces the growth of ovarian cancer cell lines [9]. SPARC is downregulated upon transformation of fibroblasts [10, 11], and upregulated with late differentiation of keratinocytes [12] and skeletal myoblasts [13]. It is prominently expressed by the retinal pigment epithelium of the eye and lack of SPARC leads to cataracts in SPARC null mice [14]. Moreover, mesangial cells, fibroblasts and aortic smooth muscle cells derived from SPARC null mice proliferate faster than wild-type counterparts [15]. SPARC can also effect cell migration. It is haptotactic for renal cell carcinoma cells in Boyden chamber assays [16], and promotes prostate and breast cancer cell chemotactic migration and invasion *in vitro* [8]. These data suggest a role for SPARC in mediating metastasis to bone by these different carcinoma types.

We previously found that SPARC was selectively expressed by a series of invasive breast cancer cell lines studied [17]. These cell lines can be distinguished from their non-invasive counterparts by a comparative loss of epithelial attributes in preference for mesenchymal features [18]. This is consistent with the preferential expression of SPARC by mesenchymal cells rather than epithelial cells, although, as mentioned above, this is not exclusive. Breast cancer cells that have undergone an epithelial to mesenchymal transition may show heightened responsivity to SPARC. Indeed, invasive human breast cancer cell lines responded to SPARC with increased activation of matrix metalloproteinase-2 (MMP-2)/gelatinase A, an extracellular protease capable of degrading basement membrane specific, type IV collagen [17]. To study further the effect of SPARC on human breast cancer cells, we engineered the MDA-MB-231 BAG cell line, which expresses very low levels of SPARC, with a DOX-inducible vector to control SPARC expression, and examined the biochemical and biological consequences. Unlike melanoma and glioma cells that respond to SPARC with increased proliferation and/or migration and invasion, the MDA-MB-231 BAG cell migration and invasion was unaffected by SPARC, which instead casued reduced proliferation.

Material and methods

Cell culture and reagents

MDA-MB-231 human breast cancer cells, originally from ATCC (Rockville, MD, USA), were genetically tagged by transduction of the bacterial β-galactosidase (Lac Z) retroviral vector to generate the MDA-MB-231 BAG cell line as previously described [19, 20]. They were cultivated in DMEM (Life Technologies, Grand Island, NY, USA) supplemented with 10% fetal calf serum (DMEM-FCS) (CSL Limited Biosciences, Parkville, Victoria, Australia). Bovine aortic endothelial (BAE) cells were used at passage 8 and also cultivated in DMEM-FCS. Cultures were maintained at 37°C in 5% CO_2. Doxycycline hydrochloride (DOX; Sigma, St. Louis, USA) was used at 2 μg/ml unless otherwise specified. Matrigel was kindly provided by Dr Hynda Kleinman, NIDCR, NIH, USA. SPARC purified from bovine bone was purchased from Haematologic Technologies Inc., VT, USA, and recombinant human SPARC purified from transfected HEK293 cells [21] was kindly provided by Prof. Rupert Timpl, Max-Plank-Institut Für Biochmie, Germany.

Plasmids

The pUHG17-1, pUHC13-3 and pUHD10-3 were kindly provided by Prof. H. Bujard, Department of Molecular Biology, University of Heidelberg, Germany [22, 23]. The pUHG17-1 plasmid contains the rtTA transactivator gene. The pUHD 10-3 plasmid contains seven repeats of the tet operator linked to a cytomegalovirus minimal promotor, upstream from multiple cloning site and a simian virus 40 polyadenylation signal. The SPARC cDNA, hon-2 [24] (kindly provided by Dr Larry Fisher, NIDCR, NIH, USA) was partially digested with *Eco*RI and subcloned into pUHD10-3 to generate pUHD10-3-SPARC. pUHC13-3 contains luciferase under rtTA responsive promotor. The pSV40Zeo plasmid encoding Zeocin resistance gene (Promega, Madison, WI, USA) and pCHC6 [25] encoding hygromycin resistance were used as selective markers for the first and second rounds of transfection, respectively. All plasmids were prepared by QIAGEN mini and maxi plasmid preparation kits (QIAGEN Pty Ltd, Victoria, Australia).

Transfection

To generate SPARC-inducible clones, we performed two rounds of transfection. In each case, the MDA-MB-231 BAG cells were plated overnight (200,000 cells per well) in 6-well-plates in DMEM-FCS. For the first round transfection, 2 µg of pUHG17-1 and 0.2 µg of pSV40Zeo were cotransfected the next day using Fugene (4 µl; Boehringer Mannheim, USA). Clones were selected in the presence of 800 µg/ml of Zeocin (Invitrogen, The Netherlands) and cloned using cloning cylinders. RNA from each clone was extracted and expression of rtTA was assessed by RT-PCR. High expression clones were further assessed by transient transfection with pUHC13-3 in the presence of 2 µg/ml of DOX for 48 h. Cell lysates were collected for analysis with the Luciferase assay system (Promega) as per manufacturers' guidelines. We also performed transient transfection with pUHD10-3-SPARC in the presence of 2 µg/ml DOX, and conditioned media were analysed for SPARC by western blot. Collectively these assays identified three clones (X, Y and Z) with the highest expression of rtTA in the presence of DOX, and the lowest in its absence.

For the second round transfection, each of the three clones (X, Y and Z) were cotransfected with 2 µg/ml of pUHD10-3-SPARC and 0.2 µg of pCHC6 using Fugene (4 µl). The clones were selected in the presence of 600 µg/ml Hygromycin B (Gibco BRL, Scotland). To rapidly screen for strong expression, clones were initially grown in the presence of 2 µg/ml DOX for 72 h and the conditioned media were collected and subjected to western analysis for SPARC. Those with high expression were further tested in the presence and absence of DOX to identify clones with the lowest baseline and highest inducibility (X5, X21, X24 and X75), which were selected for further work. High background was found in clones derived from Y, and only low levels of SPARC were achievable in clones derived from Z, so these were not used further.

Western analysis

Analyses of SPARC expression in the MDA-MB-231 BAG and transfected clones were performed on conditioned media. Cells (50,000/24 well vessel) were plated overnight in DMEM-FCS to achieve a semi-confluent monolayer, which was washed three times the next day with the unsupplemented DMEM (0.2 ml) and then incubated for up to 96 h (as specified) with DMEM supplemented only with 0.1% BSA (Sigma, cell culture grade. DMEM-BSA) with or without DOX (2 µg/ml unless otherwise stated). Proteins in the unconcentrated conditioned media were separated under reducing conditions on 10% SDS-PAGE. Loading was standardized by the amount of total protein in each cell lysate. The proteins were then transferred to PVDF membrane (Immobilon, Millipore Corp., Bedford, MA, USA) for immunoblotting. Transfer was monitored by reversible staining with Ponceau Red (Sigma). The blots were blocked for 2 h with blocking solution (5% skim milk, 0.05% Tween 20 in PBS pH 7.5) and then incubated with primary antibody (Anti-Osteonectin, Haematologic Technologies Inc., 5.4 µg/ml) in blocking solution overnight at 4°C. The membrane was then washed three times for 10 min with blocking solution and incubated for 1.5 h at room temperature with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG antibody (Pierce, IL, USA) diluted 1:20,000. Signals were developed with an enhanced chemiluminescence (ECL) kit according to the manufacturer's instructions (Pierce).

Adhesion assay

Dilutions (2.5, 1, 0.5, 0.25, 0.125, 0.05 µg/ml) of collagen types I (Vitrogen100, Cohesion, CA, USA) and IV (Sigma) were prepared in DMEM-BSA, added (50 µl per well) in triplicate to 96-well plates, and incubated for 60 min at 37°C. Wells were then washed with 100 µl of PBS containing 3% (w/v) BSA for 30 min at 37°C. Cells were harvested from routine culture with PBS containing 0.2 mg/ml EDTA, washed twice in DMEM-BSA, and resuspended at 2.5×10^5 cells/ml DMEM-BSA. The cells were incubated with occasional mixing for 60 min at 37°C to allow for recovery of cell surface receptors. Cells (2.5×10^4 in 100 µl of media) were added to each well and incubated for 60 min at 37°C. The conditioned medium was removed from each well and the attached cells stained by the addition of 100 µl of Crystal Violet (0.5% (w/v) in 25% (v/v) methanol) for 5 min at room temperature. Wells were gently rinsed five times with water to remove unbound stain and allowed to air-dry at room temperature. Even distribution of cells indicated even coating of the wells with each substrate. Bound crystal violet was solubilized with 50% (v/v) ethanol for 10–15 min at room temperature, and absorbance read at 540 nm (Power Wave X, Bio-Tek Instruments, Inc., Winooski, VT, USA).

Proliferation assay

Cells (1,000 per well) were plated in 96-well plates in DMEM-FCS (200 µl per well). Each clone was grown in the presence or absence of 2 µg/ml of DOX (5 wells/treatment). One plate was fixed each day for the next 10 days, with 50 µl per well of cold 50% (w/v) trichloroacetic acid (TCA, Sigma) for 1 h at 4°C. The conditioned medium was then discarded, and plates were washed five times with distilled water and air-dried. After 10 days, all 10 plates were stained with sulforhodamine B solution (SRB, 0.4% w/v in 1% acetic acid; 100 µl per well) (Sigma), and incubated for 10 min at room temperature. Unbound SRB was removed by washing five times with 1% acetic acid, the plates were air-dried, bound stain was solubilized with 10 mM Tris-buffered saline and the optical densities read at 515 nm. Statistical analysis was performed using the General Linear Model program in SPSS (Chicago, Illinois, USA) and Linear Regression Analysis in GraphPad Prism 3 (GraphPad Software, San Diego, CA, USA).

Cell cycle analysis

Cells (2.5×10^5) were seeded in a 10 cm plate with or without 2 µg/ml of DOX in DMEM-FCS. The medium was replaced 48 h later with fresh DMEM-FCS with the continued presence or absence of DOX. After an additional 24 h, the cells were trypsinized (0.025%, CSL Ltd., Victoria, Australia) washed with PBS-BSA and fixed in 1 ml of paraformaldehyde at 4°C for 10 min. Then the cells were collected by centrifugation (1500 rpm for 5 min), resuspended in 1 ml of 0.1% Triton X-100 and incubated at 4°C for 10 min. The cells were again centrifuged and resuspended in 1 ml of solution containing 50 µg/ml propidium iodide (Sigma), 0.1% sodium citrate and 1 µg/ml RNase. The stained cells were analyzed within 24 h on FACS Calibur (Becton Dickinson, USA).

Monolayer wound healing assay

Cells (50,000 cells/well) were plated in 24-well plates in DMEM-FCS and grown to approximately 80% confluency. The monolayer was wounded by scraping a line across the well using a sterile 1 ml blue pipette tip, after which the culture was washed twice with DMEM and the media changed to DMEM-BSA with or without 2 µg/ml of DOX and in some cases, with or without 10 mM thymidine (Sigma). The monolayer wound was photographed every days from days 0 to 4 at the same spot, guided by markings under the plate. The width of the monolayer wound was measured from each photograph and the closure expressed as percent day 0. Statistical analysis was performed with the General Linear Model program in SPSS.

Chemomigration assay

Cell migration was determined using a 48-well microchemotaxis chamber assay (Neuroprobe, Cabin John, MD, USA) as described previously [20]. Cell migration was quantified by the number of cells that migrated directionally through a collagen I (10 µg/ml) coated 8 µm pore polyvinyl pyrrolidone-free polycarbonate filter (Poretics, Livermore, CA, USA) toward the chemoattractant. Briefly, cells (1×10^6 cells/ml), either pretreated or untreated with 2 µg/ml of DOX for 48 h, were resuspended in DMEM-BSA and loaded into the top chamber. Fibroblast conditioned medium was used as the chemoattractant and loaded into the bottom chamber. Chambers were incubated at 37°C in 5% CO_2 for 4 h, after which the filters were removed, fixed and stained with Diff-Quik (Baxer Scientific, McGaw Park, IL, USA) and mounted on glass slides. Nonmigrated cells were removed by wiping with a cotton swab. At least four random fields of vision/well (×20 objective) were counted for quantitation of cell migration. Triplicate wells were used in each assay.

Matrigel outgrowth and radial outgrowth

In vitro invasion analysis was performed with a novel adaptation of the Matrigel outgrowth assay [26]. Briefly, cells were trypsinized, counted, centrifuged and resuspended in Matrigel to give densities of 250, 500 and 1000 cells per 5 µl of Matrigel. Five microlitres of Matrigel containing each cell line or clone was placed in each well (96-well ELISA plate) and let set in an incubator at 37°C in 5% CO_2 for 1 h. After that, 80 µl of DMEM-FCS with or without 2 µg/ml of DOX were added on top of the droplet. For radial outgrowth, after the Matrigel droplet was set, 80 µl of 2 mg/ml collagen type I (Vitrogen100) was overlaid and incubated at 37°C in 5% CO_2 for 1 h. This was followed by 80 µl of DMEM-FCS with or without 2 µg/ml of DOX. Cell morphology was observed and photographed daily for up to 7 days.

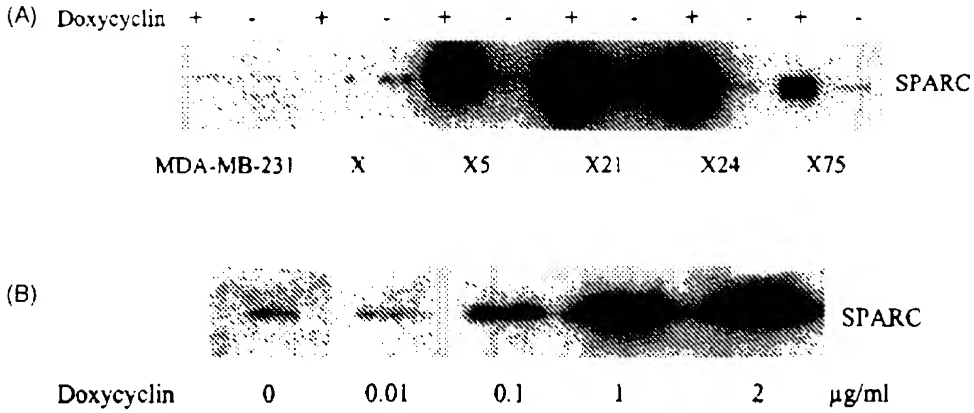


Figure 1. (A) Inducible expression of human SPARC in breast cancer cells. The MDA-MB-231 BAG parental, clone X parental, clone X5, X21, X24 and X75 (50,000) were seeded in 24-well plates in the presence and absence of 2 μg/ml DOX for 96 h. The conditioned media were then collected and subjected to western analysis. (B) DOX concentration-dependent induction of SPARC expression in clone X5. Cells (50,000) were seeded in 24-well plates as described in Materials and Methods, and treated with of 0, 0.01, 0.1, 1 and 2 μg/ml DOX for 24 h. The conditioned media were collected and subjected to western analysis. The amount of SPARC expression correlates with the concentration of DOX used.

Results

Detection and regulation of SPARC protein expression

To evaluate the level of SPARC protein expression in MDA-MB-231 BAG cells, clone X parental cells and transfected clones X5, X21, X24 and X75, we performed western analysis of serum-free conditioned media generated in the presence or absence of 2 μg/ml of DOX. Previously we found SPARC to be expressed in invasive breast cancer cell lines, BT-549 and Hs578T but not in MDA-MB-231 BAG by northern analysis [17]. Here, we found low levels of SPARC in MDA-MB-231 BAG cultures after 4 days, and the level of expression did not change after DOX treatment (Figure 1(A)). A similar level of expression was also found in clone X parental cells prior to SPARC transfection. After SPARC transfection, basal levels of expression in clones X5, X24 and X75 remained low but could be highly induced after DOX treatment. Clone X21 showed somewhat higher basal expression of SPARC in the absence of DOX, but this level could still be dramatically increased by DOX.

Addition of a range of DOX concentrations from 0.01 to 2 μg/ml to clone X5 induced concentration-dependent expression of SPARC (Figure 1(B)) while higher concentrations up to 8 μg/ml showed no increased expression (data not shown). Comparative western analysis with commercial bovine SPARC at the stated concentration allowed estimation of the SPARC accumulation from clone X5 after 3 days of DOX-treatment (2 μg/ml) to be around 5 μg/ml (data not shown).

SPARC has no effect on morphology of transfected clones or on their attachment to collagens type I and IV

Despite pronounced effects on 'rounding' of endothelial cells, smooth muscle cells and fibroblasts [5], we did not observe any changes in cell morphology after DOX treatment of our SPARC-transfected clones. Representative clones (X5, X24, X21 and X75, as well as parental clone X, or MDA-MB-231 BAG cells) were stained with crystal violet [27] after treatment with or without DOX (data not shown). Although each clone adopted a slightly different morphology, with some better spread than others, none responded morphologically to the induced SPARC. Similarly, addition of up to 100 μg/ml exogenous, purified or recombinant SPARC did not alter MDA-MB-231 BAG cell morphology, but did induce rounding of bovine aortic endothelial cells (data not shown). SPARC is known to have an anti-adhesive effect in certain cell types, achieved in part by the dissolution of focal adhesion complexes and reorganization of actin stress fibers [3]. Neither parental MDA-MB-231 BAG cells, parental clone X or transfected clones showed any changes in cell attachment to collagen type I, after pre-treatment with DOX for 3 days (Figure 2). Concentration-dependent increases in attachment were seen from 0.05 μg/ml up to 2.5 μg/ml

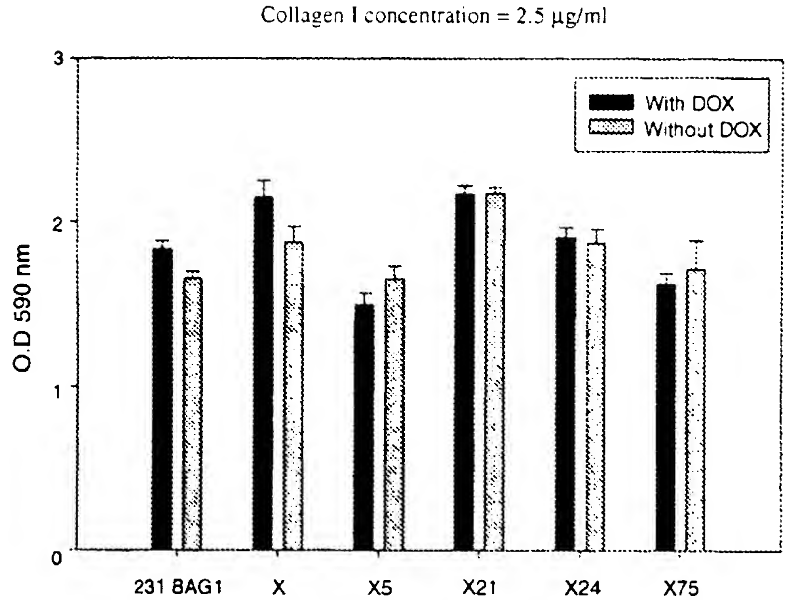


Figure 2. SPARC expression has no effect on cell attachment to collagen I coated plates. The MDA-MB-231 BAG, clone X parental, clone X5, X21, X24 and X75 were plated on collagen I coated plate in triplicate and analyzed for attachment after 1 h as described in Material and methods.

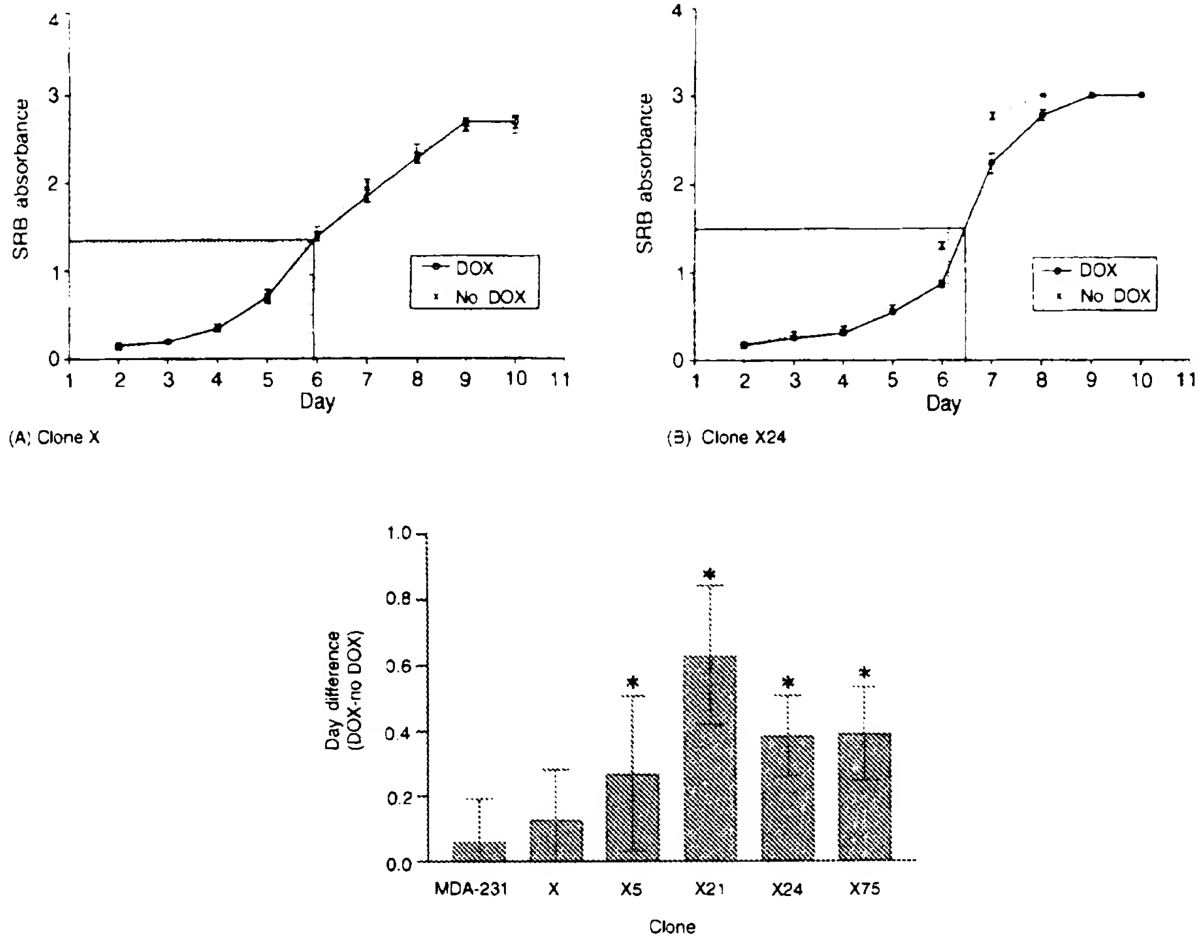
collagen type I for each clone, but there was no difference between the DOX-treated and untreated group (data not shown). Similarly, no difference was found between the two groups in adhesion to collagen type IV, a substrate to which the cells attach with a lower affinity (data not shown). We further tested the effects of SPARC-enriched (+DOX) or control (−DOX) conditioned medium on the subsequent attachment of these cells to either collagen type I or type IV, but no difference was seen (data not shown). SPARC does not appear to affect the adhesion of these cells to collagens type I or IV.

SPARC inhibits anchorage-dependent proliferation of transfected clones

We then looked at the effect of DOX-induced SPARC on proliferation of the transfected MDA-MB-231 BAG cells as numerous studies have reported differential effects of SPARC on cell proliferation. In the control MDA-MB-231 BAG parental (not shown) and clone X parental (Figure 3(A)) cells, DOX had no effect on growth. The cells grew to confluency over the 10-day period in both DOX-treated and untreated groups. However, for each of the SPARC-transfected clones, the DOX-treated group showed a slower rate of proliferation (shown in Figure 3(B) for clone X24, data not shown for clones X5, X21 and X75). DOX treatment of all SPARC-transfected clones caused a delay in reaching half maximal growth (Figure 3(C)), but each clone finally reached the same confluency. DOX was only added on the first day of the experiment so as to avoid media changes and allow the cultures to accumulate SPARC. A more pronounced inhibition may have been seen with repeated addition of DOX, but this was technically difficult. Nonetheless, this small but significant growth inhibition was reproducible and consistent between five replicates of each group in three independent experiments.

Cell cycle analysis

To analyse the growth effect further, we grew the MDA-MB-231 BAG parental (Figure 4, (A), (B)), parental clone X (not shown), clone X5 (Figure 4(C)–(F)) and clone X24 (data not shown) in the presence or absence of DOX and harvested them for analysis of cell cycle distribution. No effects of DOX were seen on the MDA-MB-231 BAG or parental clone X cells, but both clone X-5 and X-24 showed an increase in S-phase after DOX treatment. For clone X5, increasing concentrations of DOX from 0 to 2 μg/ml showed a concentration-dependent reduction of the proportion of cells in S-phase.



(A) Clone X

(B) Clone X24

(C) Half maximal growth differences

Figure 3. *In vitro* proliferation analysis of parental and transfected clones. Cells were seeded in 96-well plates in DMEM-10% FCS with or without 2 μg/ml of DOX. Proliferation was measured every day as described in Material and methods. (A), (B) Growth curves of clone X parental and clone X24, respectively. Solid line indicates the DOX treated group and the dotted lines indicate the untreated group. In (B), statistical analysis using General Linear Model showed a significant difference in growth kinetics ($P < 0.001$). (C). The difference in time when half maximal growth was reached in the presence compared to the absence of DOX. Ninetyfive percent confidence intervals are shown. * denotes significant difference ($P < 0.05$).

DOX-induced SPARC slows down monolayer wound healing but has no effect on cell migration or Matrigel outgrowth

To assess the composite effects of SPARC on cellular remodelling *in vitro*, we performed the cultured monolayer wound healing assay over 4 days. In this assay, closure of the monolayer wound depends on both proliferation and migration of the cells. Since SPARC is secreted and present in the conditioned media around the cells, it might work chemokinetically as reported in melanoma cells [7]. As shown in Figure 5, however, clone X5 showed a slower rate of monolayer wound closure when SPARC expression was stimulated with DOX. Similar changes were seen in clones X21, X24 and X75 (data not shown) while clone X parental consistently showed no response to DOX. Also, there was no difference between the DOX-treated and untreated group in the MDA-MB-231 BAG parental cells, which reproducibly showed a quicker rate of closure than the other clones (data not shown). To delineate the proliferative component, we performed this assay in the presence of 10 mM thymidine, which blocks cell proliferation (Figure 5(C) inset). As shown in Figure 5(C), we found no effect of DOX on the rate of closure in the presence of thymidine, and further-

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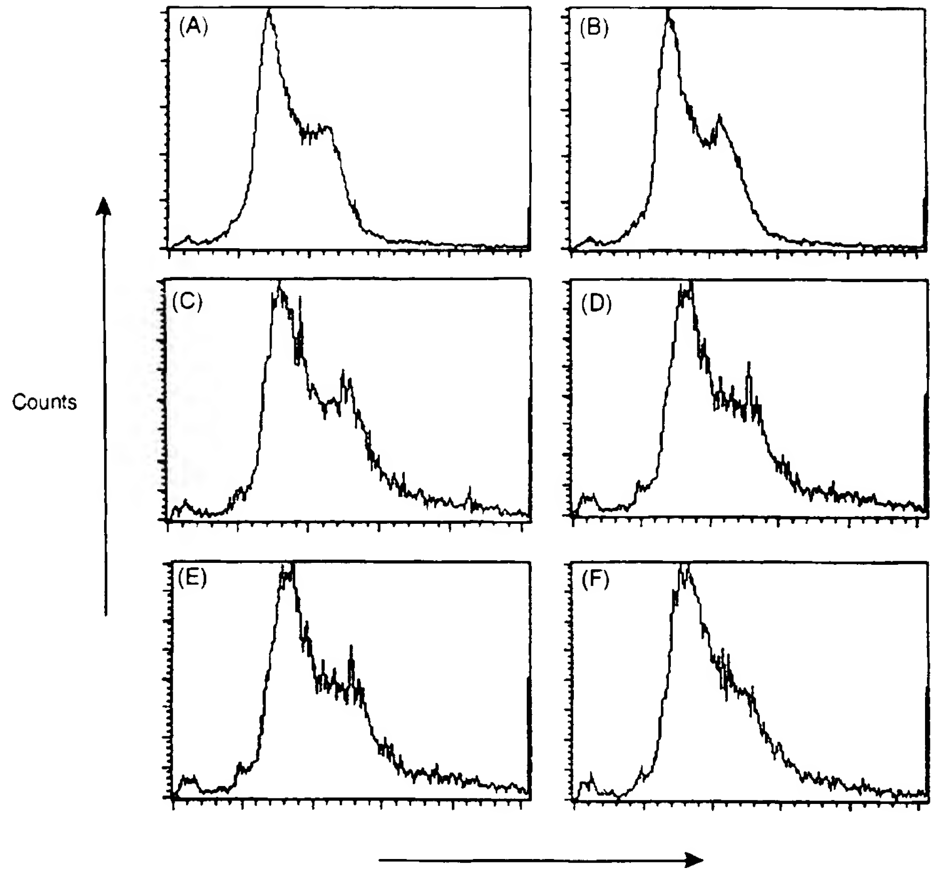

Figure 4. Cell cycle analysis of representative clones. The MDA-MB-231 BAG (A), (B) and clone X5 (C)–(F) were cultured in the presence or absence of DOX (0.02, 0.2 or 2 µg/ml) for 72 h prior to cell cycle analysis by flow cytometry.

more, the capacity for monolayer wound closure was returned to non-DOX levels. These data confirmed the anti-proliferative effect of SPARC, and suggest that there is no chemokinetic effect of SPARC in our system.

Consistent with this, we were unable to detect any differences in the 48-well microchemotaxis migration of DOX-treated versus untreated clones towards fibroblast conditioned medium over 4 h on collagen type I coated filters, even when the conditioned media from each culture was added into the chamber (data not shown). Similarly, long term culture (up to 7 days) in 3-dimensional gels of collagen or Matrigel did not show any differences (data not shown), and no differences were seen in the ability of the cells to penetrate the collagen matrix in the radial outgrowth assay (data not shown).

Discussion

SPARC is a secreted protein which is abundant in tissues undergoing remodelling, and has been found to have dramatic effects on cell behaviour *in vitro*. First purified as a major non-collagenous component of bovine bone [2], its biological significance was linked to the regulation of bone mineralization. Later studies also showed expression of SPARC in non-mineralized tissue including gut, skin, liver, vascular smooth muscle cells and platelets [28–31].

SPARC is overexpressed in many cancers. Malignancies of mesenchymal origin consistently exhibited strong immunostaining of SPARC [28]. It is overexpressed during neoplastic progression of human melanoma [32], meningiomas [33], and glioma [34], and is associated with invasiveness. Many

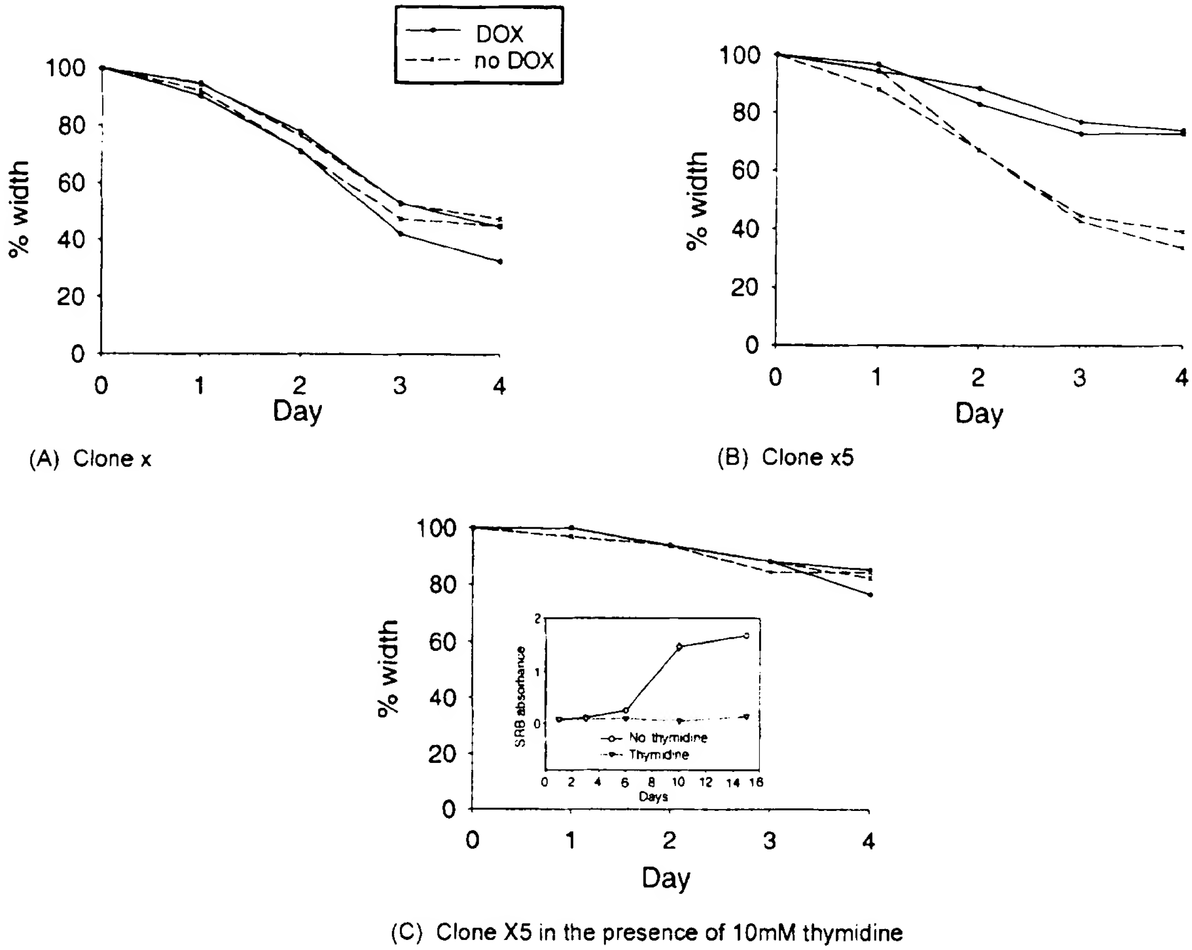

Figure 5. Monolayer wound healing assay of clone X parental (A), clone X5 (B). Clone X5 was also tested in the presence of 10 mM thymidine (C). Inset in C shows the abrogation of the MDA-MB-231 BAG cell proliferation by 10 mM thymidine. Cells were plated and monolayers wounded in the presence and absence of 2 μg/ml DOX. A photograph was taken each day for a period of 4 days and the width of the monolayer wound was measured. The starting size of each monolayer wound was designated as 100%. Data are presented from duplicate wells in one representative experiment of three independent experiments performed. In (B), statistical analysis by General Linear Model showed significant differences in monolayer wound closure between the DOX-treated and untreated cells (P-value = 0.008).

epithelial tumours exhibit high levels of SPARC [28], including esophageal carcinoma [35], hepatocellular carcinoma [36], prostate carcinoma [37] and breast carcinoma [38, 39]. Despite intensive immunostaining of SPARC in the carcinoma parenchyma, *in situ* hybridization studies show that SPARC is usually produced by the surrounding stromal cells. This appears to be the case for breast [39] and hepatocellular [36] carcinoma, but both stromal and parenchymal cells were found positive in prostate carcinoma [37]. Ovarian carcinoma is rather unique, since the normal ovarian surface epithelium is positive for SPARC expression, and this is lost from ovarian carcinoma [9].

In vitro, SPARC is expressed in cancer cell lines from many origins, including glioblastoma [27], melanoma [7] and prostate cancer [37]. For breast cancer cell lines, our previous work showed SPARC not to be expressed by better-differentiated lines like T47D and MCF-7, but expressed in cell lines that have acquired mesenchymal features such as BT-549, MDA-435 and Hs578T [17]. In the present study, we further found that the mesenchymal-like MDA-MB-231 BAG cells also expressed SPARC, but at a lower level. This is consistent with its expression *in vivo* by carcinoma-associated fibroblasts and by tumour cells of mesenchymal origins. The low levels of SPARC produced by the MDA-MB-231 cells, and their

continued co-expression of keratin as well as vimentin [40], suggest that they may show behavioural tendencies of both epithelioid and mesenchymal cells, with respect to SPARC responsivity. Further analysis in a panel of breast cancer cell lines will be required to answer this question more fully. Approximately 10–15% of breast tumours show vimentin expression as a putative indicator of mesenchymal *trans*-differentiation [18], however, SPARC expression has not been examined in these.

SPARC has various biological functions relevant to soft tissue remodelling, including cell migration, proliferation and angiogenesis [5, 8, 16, 41, 42]. It has also been shown to affect morphology in a number of cell systems, particularly normal mesenchymal cells. It causes rounding of bovine aortic endothelial cells (BAE), fibroblasts and smooth muscle cells [5]. SPARC-null mesangial cells exhibit a flat morphology and an altered actin cytoskeleton, with vinculin-containing focal adhesions distributed over their center. Although SPARC-null fibroblasts did not display any overt differences in cell morphology, they responded to exogenous rSPARC by rounding up in a manner similar to wild-type fibroblasts [15]. In contrast, the U87MG glioblastoma cells transfected with SPARC displayed a flatter morphology with extended cytoplasm instead of being round as expected [27]. In the current study, however, we did not see any morphological effects of the induced SPARC on the MDA-MB-231 BAG cells either in monolayer culture or on 3-dimensional Matrigel, nor when we added exogenous SPARC in cell culture. Induction of SPARC also appeared not to cause any change in adhesion of the cells to either type I or type IV collagen, or migration over filters coated with type I collagen, however, additional ECM substrates which are found in breast cancers (e.g., fibronectin, vitronectin, proteoglycans) were not tested. It has been noted that SPARC effects on cell morphology are less apparent, or absent, in transformed cells [43].

Many of the reported effects of SPARC on cancer cells can be associated with poor outcome. U87 glioblastoma cells transfected with SPARC using tetracycline-inducible gene expression (Tet-Off) showed altered adhesion and increased invasion *in vitro* [27]. In a melanoma cell line, suppression of SPARC expression using antisense RNA significantly decreased *in vitro* adhesive and invasive capacities, and completely abolished *in vivo* tumourigenicity [7]. Additionally, a positive role of SPARC in the process of angiogenesis has been indicated [44]. In contrast, our transfected clones showed no increased migration or invasion *in vitro* in a variety of assays. This could be due to their carcinomatous nature, as ovarian carcinoma cells were also shown to be suppressed rather than stimulated by SPARC [9]. Different response profiles may be due to different SPARC receptors between cell types. Although cell-surface proteins have been shown to bind specifically to SPARC, there is no reported or characterized receptor for SPARC [4]. SPARC has also been shown to induce chemotactic migration of the MDA-MB-231 cells [8] but we did not assess this type of migration here.

With our previous work, we found that SPARC could induce MMP-2 activation in invasive breast cancer cell lines BT-549 and MDA-MB-231. However, we were unable to see this effect with DOX-induced SPARC expression in our clones. We estimated the SPARC concentration accumulated by our clones after DOX induction to be around 5 μg/ml, and this level was not high enough to elicit MMP-2 activation effect in the previous study [17], where 25–50 μg/ml SPARC was required. While these levels appear high, they may well be achieved in the bone environment, and could also reflect levels achieved by adsorption of SPARC to collagen matrices. Attempts to develop further accumulation of SPARC in our DOX-induced cultures, by long-term culture (up to 10 days) in 3-dimensional matrices collagen and Matrigel, did not lead to MMP-2 activation.

SPARC has been shown to variably effect cell proliferation. In some cell types, SPARC seems to have no effect on growth while in others it is anti-proliferative [7–9]. We found an anti-proliferative effect in all of the transfected clones tested. This effect was not large but was highly reproducible. The cultured monolayer wound healing assay also confirmed the anti-proliferative effect of SPARC in the transfected clones, since the effect was abrogated when proliferation was blocked with thymidine. This inhibition is consistent with that seen in ovarian carcinoma cells [9], and perhaps also with the increase in SPARC with differentiation of various systems and its loss from certain systems upon transformation [43]. We did not, however, specifically test whether cell density had any effect on the anti-proliferative responses, other than the two densities used for the cell proliferation and monolayer wound healing assays. Differentiated embryonic carcinoma cells grown at high densities express higher levels of SPARC than their low density counterparts [45], and the *in vitro* growth of melanoma cells in which SPARC was down-regulated by

antisense expression also show attenuation with the density of the seeded cells [7]. Perhaps in a similar light, angiogenenic endotheial cells respond to SPARC whilst non-angiogenis cells do not [46]. These considerations illustrate the complexity of SPARC biology, and emphasises the need for further analysis of SPARC effects in different cell systems.

We do not know whether this anti-proliferative effect is direct or indirect, since SPARC has been found to associate with certain growth factors. It can bind to vascular endothelial growth factor (VEGF) and inhibit VEGF-stimulated proliferation of human microvascular endothelial cells [47], and can counteract the proliferative effect of basic fibroblast growth factor on smooth muscle cells [48]. DNA staining and cell cycle analysis showed a decreased proportion of cells in S-phase in a DOX concentration-dependent manner. This suggests a role of SPARC on cell cycle progression to S-phase in breast cancer cell line, as has been reported for endothelial cells [6]. Although chemically modified tetracyclines can inhibit MDA-MB-468 human breast cancer cell proliferation shown by slower rate of closure in a cultured monolayer wound healing assay [49], we never saw this effect on our parental cells with up to 2 μg/ml DOX and conclude that the effects that we saw were due to SPARC, not DOX.

In conclusion, the effect of SPARC on breast cancer cells reported here appears quite different to that seen in melanoma or glioma cell lines. SPARC did not effect cell morphology, adhesive properties, migration or invasion, but rather inhibited proliferation. It is possible then that SPARC is a beneficial host factor in breast cancer, unlike what has been reported in melanoma, meningioma and glioma where SPARC seems to induce an invasive phenotype. Indeed, the higher levels of SPARC associated with more advanced breast cancers [38], and the inverse correlation was seen between SPARC mRNA expression and estrogen receptor levels in breast tumour biopsies [50] may represent an increased host effort to combat the carcinoma. The previous interpretation has been that the higher levels of SPARC seen in association with more advanced breast cancers indicated an adverse role for SPARC. However, the host may simply be making more SPARC in response to more pronounced cancer-derived signals, in a protective manner. The results reported here indicate that further analysis of the biological consequences of SPARC in breast cancers is warranted.

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